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INTRALYMPHATIC IMMUNOTHERAPY IN ALLERGIC RHINITIS - EVALUATING SAFETY, EFFICACY AND MECHANISMS

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Intralymphatic immunotherapy in allergic rhinitis – Evaluating safety, efficacy and mechanisms

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To Max

”Men alltid reste sig ett nytt krön i hans synfält. Oändlighetsfjäll. Stenvåg efter stenvåg. Dvärgbjörk och kråkris. Här och där en trädknota med stelnad vindjämmer i. Varenda fjällbjörk var vriden som en utsliten käring. Vilket jävla liv. Vilket vindplågat ljussnålt marigt helvete.”

Kerstin Ekman ur Sista rompan

“It’s a universal law- intolerance is the first sign of an inadequate education. An ill-educated person behaves with arrogant impatience, whereas truly profound education breeds humility.”

from August 1914 by Aleksandr I. Solzhenitsyn
Translated by H.T. Willetts

ABSTRACT

Allergic rhinitis (AR) deprives work capacity, social activities and quality of life, and costs the Swedish society about €1.3 billion annually. Allergen-specific immunotherapy (AIT) amends the symptoms and improves the course of the disease. The symptom ameliorating effects last several years after the discontinuation of treatment. The golden standard for immunotherapy of AIT is subcutaneous administration but during the last decade sublingual immunotherapy has become common. Both forms of AIT are underused due to the lack of knowledge about the treatments among physicians, lack of access to the treatment and inconvenience for the patients. Intralymphatic immunotherapy (ILIT) is an emerging form of AIT, which requires only 3 injections during a period of 8 weeks.

The aim of this thesis was to evaluate the intralymphatic route by using different treatment protocols and to characterize immunological signs of tolerance development.

In paper I, asthmatic young adults were treated in a randomized double-blind placebo-controlled (RDBPC) trial with three intralymphatic injections of birch or grass allergen in doses of 1000 SQ-U, or placebo. The active group returned the next year for a booster injection. The treatment was safe, even in patients with mild asthma. The use of symptom relieving medications at the pollen season was reduced the first and the second year after treatment. The allergen specific IgG4 antibodies were increased 6-9 months after treatment. The asthma symptoms could not be improved among well-treated patients.

In paper II, polysensitized patients received ILIT for both birch and grass induced AR in a RDBPC trial, with doses of 1000 SQ-U each, in three injections. The treatment was safe, even with two allergens given simultaneously. The rhinoconjunctivitis symptoms after a nasal provocation test were improved 6-9 months after treatment and the use of symptom relieving antihistamines and/or nasal steroid spray was reduced. The timothy specific IgG4-levels, regulatory T-cells (Tregs) and Th1 type of T-cells were increased in blood and the effector memory T-cells were increased in the lymph nodes after treatment.

In paper III, ILIT in up-dosing schedules were evaluated in two RDBPC trials. In ILIT after SCIT-10 000, patients that had recently received SCIT for grass AR, were treated with 1000- 3000- 10 000 SQ-U of grass allergen, with one-month intervals. The treatment was safe. The combined symptoms and medication scores (CSMS) were improved during the pollen season after treatment and the timothy specific IgG4 levels were increased. In ILIT de novo- 3000, patients with grass induced AR without previous AIT were recruited. The dose-escalation was safe up to 3000 SQ-U, but serious anaphylactic reactions occurred at 5000 SQ-U. The patients that were treated with the modified protocol 1000-3000-3000 SQ-U did

not improve the AR symptoms at pollen season and had no clear signs of beneficial immunologic changes in blood or lymph nodes.

In paper IV, the patients that were treated with active ILIT 5-6 years previously in paper II, returned for an open follow-up and were compared to a non-AIT treated control group. The symptoms at NPT were unchanged, but the CSMS at the pollen season was lower compared to in the control group. Timothy specific IgE levels had decreased markedly compared to before treatment, IgG4 was still slightly elevated and the lymph node samples displayed increased levels of memory T-cells.

In summary, ILIT with 1000 SQ-U was safe with mild asthma and when given with two allergens concomitantly. Up-dosing to 10 000 SQ-U was safe among previously SCIT-treated patients, but dose-escalation to 5000 SQ-U induced anaphylaxes in de-novo patients and should not be performed. The AR symptoms were improved after 1000 SQ-U and after up-dosing to 10 000 SQ-U among SCIT-treated patients, but up-dosing to 3000 SQ-U failed to improve the clinical symptoms in previously unvaccinated patients. Early immunological changes included increases in timothy specific IgE and IgG4 levels, Treg and Th1 levels in blood and an increase in the number of EM T-cells in lymph nodes. Long term changes noted were reduced specific IgE levels in blood and an increased number of memory T-cells in lymph nodes, as signs of a possible long-term treatment effect.

LIST OF SCIENTIFIC PAPERS

I. Intralymphatic immunotherapy in pollen-allergic young adults with rhinoconjunctivitis and mild asthma: A randomized trial

Konradsen JR, Grundström J, Hellkvist L, Tran TAT, Andersson N, Gafvelin G, Kiewiet MBG, Hamsten C, Tang J, Parkin RV, Shamji MH, Hedlin G, Cardell LO, van Hage M

J Allergy Clin Immunol. 2020 Mar;145(3):1005-1007.e7. doi: 10.1016/j.jaci.2019.11.017. Epub 2019 Nov 24.

II. Intralymphatic immunotherapy with 2 concomitant allergens, birch and grass: A randomized, double-blind, placebo-controlled trial

Hellkvist L, Hjalmarsson E, Kumlien Georén S, Karlsson A, Lundkvist K, Winqvist O, Westin U, Cardell LO

J Allergy Clin Immunol. 2018 Oct;142(4):1338-1341.e9. doi: 10.1016/j.jaci.2018.05.030. Epub 2018 Jun 13.

III. High dose grass pollen intralymphatic immunotherapy: two RDBPC trials question the benefit of dose increases

Hellkvist L, Hjalmarsson E, Weinfeld D, Dahl Å, Karlsson A, Westman M, Lundkvist K, Winquist O, Kumlien Georén S, Westin U, Cardell LO

Manuscript

IV. A five-year open follow up of a randomized double-blind placebo-controlled trial of intralymphatic immunotherapy for birch and grass, reveals remaining beneficial effects

Hellkvist L*, Hjalmarsson E*, Karlsson A, Winquist O, Kumlien Georén S, Westin U, Cardell LO

Manuscript

*These authors contributed equally to this work

LIST OF ABBREVIATIONS

ACT	Asthma control test
AIT	Allergy immunotherapy
AQLQ	Asthma Quality of Life Questionnaire
AR	Allergic rhinoconjunctivitis
AUC	Area under the curve
CSMS	Combined symptoms and medication score
DC	Dendritic cell
F_{ENO}	Fraction of exhaled NO
FEV_1	Forced expiratory volume in 1 second
FSC	Forward scatter
fTh	Follicular T helper cell
GAM	Generalized additive model
IFN-γ	Interferon-γ
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL	Interleukin
ILIT	Intralymphatic immunotherapy
MFI	Median fluorescence intensity
MPL	Monophosphoryl lipid A
MHC	Major histocompatibility complex
mITT	Modified Intetion To Treat
MS	Medication score
NPT	Nasal provocation test
PBMC	Peripheral blood mononuclear cells
PD_{20}	Provocative dose causing a 20% decline in FEV_1
PNIF	Peak nasal inspiratiory flow
RR	Relative risk
RQLQ	Rhinoconjunctivitis Quality of Life Questionnaire
RDBPC	Randomized double-blind placebo-controlled
RTSS	Rhinoconjuntivitis total symptom score
SCIT	Subcutaneous immunotherapy
SLIT	Sublingual immunotherapy
SPT	Skin prick test
SSC	Side scatter
Th cell	T helper cell
Treg cell	Regulatory T cell
VAS	Visual analogue scale

CONTENTS

1 AIMS

Intralymphatic immunotherapy (ILIT) has been proposed as a fast and safe alternative to conventional allergy immunotherapy. The intention of this thesis was to evaluate the intralymphatic route by using different treatment protocols and to characterize the immunological changes induced. More specific to:

- Study the safety of ILIT in three new situations; among young adults with mild asthma, when using two concomitant allergens and when increasing the doses of the allergens given
- Evaluate the effects of ILIT on allergic asthma
- Assess the clinical symptom improvements in a one-seasonal perspective, using two concomitant allergens
- Explore if an increase of the doses given improves the therapeutic outcome
- Investigate if the positive effects of ILIT remain 5-6 years after the vaccination
- Characterize immunological changes that could signal induction of tolerance

2 INTRODUCTION

2.1 Allergic rhinoconjunctivitis

The incidence of allergic rhinoconjunctivitis (AR) is increasing and today up to 40% of the younger population in westernized countries are affected. This increase has not yet shown signs to decline and the reasons for this almost epidemic evolvement are not fully understood. Worldwide around 500 million people are affected (1). Symptoms include runny and blocked nose, sneezing, nasal and ocular itching, red and watery eyes. In addition, although often not as well acknowledged, these patients suffer from inflammatory fatigue as well as tiredness due to impaired sleep (2).The disease substantially impacts the patients' quality of life and capacity at work or school (1, 3). Further, the disease is costly for the society. In Sweden, a population of less than 10 million inhabitants, it has been estimated to costs €1.3 billion annually (4). The surprisingly high cost is not only due to absenteeism, but to a large extent also due to low performance while at work (presenteeism).

AR is an IgE (immunoglobulin E) -mediated disease that is caused by B-cells overproducing IgE antibodies that reacts upon common environmental antigens called allergens. Allergens are most often proteins in e g pollen or food. IgE gets attached to the surfaces of mast cells and basophil cells that often reside in nasal and ocular tissue. When IgE antibodies on the cells encounter its specific allergen a signal through the receptor FcεR1 results in a discharge of allergic mediators in the tissue, e g histamines. This causes local symptoms such as itching and swelling with rapid onset, called the immediate reaction. The histamine and other mediators cause a late-phase allergic reaction with recruitment of other inflammatory cells, such as T-cells and eosinophils, to the site (1).

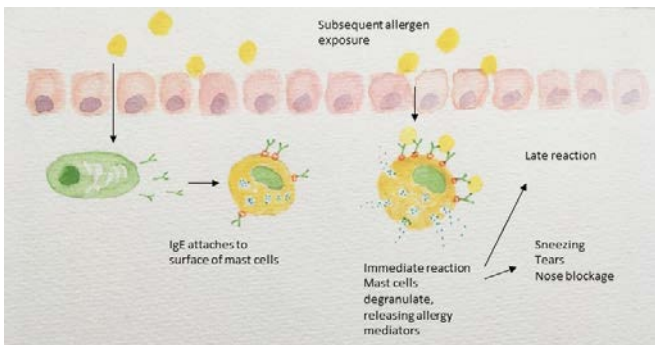


Figure 1. Sensitization at the development of AR. By unknown mechanisms, plasma cells start to produce IgE antibodies toward harmless substances, e g pollen. The IgE antibodies are secreted into blood and get attached to mast cells in the mucosa. At a later encounter with the allergen, the IgE antibodies crosslink their receptors on the mast cells. This activates the cell and causes degranulation of preformed vesicles containing e g histamine, which causes rhinoconjunctivitis symptoms at the immediate reaction. At the late reaction, inflammatory cells migrate to the mucosa and cause inflammation.

2.2 Seasonal allergic rhinoconjunctivitis

Seasonal AR is caused by intermittent exposure to allergens with duration of symptoms per definition not exceeding one month (1). However, in Northern Europe, a poly-allergic patient can experience peak allergic symptoms in May for birch pollen and later in June for grass pollen, with sometimes overlapping seasons and therefore, fulfilling criteria for perennial AR. In fact, the pollen season starts already in February with birch cross-reactivity to alder and hazel trees. If allergic also to ragweed, allergen exposure can proceed to the end of October. There are increasing reports that due to climate changes and urbanization the flora is changing. This causes spreading of allergens such as cedar tree in South East Asia (5) and ragweed in Northern America (6). Climate changes have been the cause of a prolonged vegetation period in tempered regions and therefore a longer period of allergen exposure (7).

First line treatment for mild AR is antihistamines or leukotriene receptor antagonists which often give good symptom relief (8). If the patients experience troublesome symptoms the disease is classified as moderate or severe according to the ARIA guidelines (1). Intranasal corticosteroids should then constitute the base of the treatment. However, even when adding cromoglycates or ipratropium, many patients still do not come near full symptom relief. Systemic steroids offer reliable but temporary halt of allergic symptoms and are considered as the last “rescue” option (1). Anti-IgE treatment with Omalizumab® improves symptoms effectively and has an acceptable safety profile but will not likely become a widespread treatment due to its high cost (9). Hence, there is an urgent need for development of new effective treatments.

2.3 Allergy immunotherapy

Today, the only causative treatment for AR is allergy immunotherapy (AIT). The therapy was first described in 1911 when Noon and Freeman inoculated extracts of grass pollen in order to allow the immune system to develop tolerance to the allergen (10). Today the extracts used for treatment are standardized with a defined concentration of the major allergens often administered in the form of subcutaneous injections (SCIT) and sublingual tablets (SLIT).

2.3.1 SCIT

The golden standard for the treatment of AR in Europe is subcutaneous immunotherapy (SCIT) (11, 12). It should be considered for moderate to severe AR (1). Studies have demonstrated that SCIT improves nasal/ocular symptoms and reduces the need for medication. The improvement of symptoms has been described to last up to 8 years after the end of the vaccination period (13). SCIT also improves

quality of life (13) and prevents progression of AR to asthma, at least in the short perspective (14-16). Some studies have indicated prevention of additional sensitizations (17, 18) but recently the evidence for long term protective effect has been debated (19).

The allergen that is identified as the trigger of the AR symptoms is injected subcutaneously, usually in the upper arm. The injections are repeated with increasing doses every 1-2 weeks during an up-dosing phase of 7-15 injections. The patients return for maintenance injections every 6-8 weeks during 3-4 years (13). This means SCIT is a time-consuming treatment which is inconvenient for the patients. Furthermore, even though SCIT is considered to be a cost saving treatment in the long run, with decreased loss of workdays and lower drug costs after therapy, the treatment is costly for the healthcare providers(20). In practice, it is considered only for patients with severe allergic symptoms for allergens that cannot be avoided and when conventional pharmacological treatment is insufficient. The allergen injections also convey a risk of allergic reactions such as local erythema, oedema and pruritus at the injection site, airway obstruction, nasal or ocular symptoms or urticarial rash. Anaphylactic reactions are rare but can occur (21), which is why the treatment in Sweden is preferably given at hospitals (22).

2.3.2 SLIT

During the last decades, sublingual administration (SLIT) has been developed as an alternative to SCIT. The patient takes one tablet under the tongue every day for three years. Advantages are that the risk of allergic side effects and the need for medical supervision are substantially lowered (23). Indirect comparisons between SCIT and SLIT suggest that the clinical effect of SLIT is at least close to the effect of SCIT (24). A disadvantage of SLIT is that the treatment time still lasts for 3 years, which understandingly causes problem with long term patient adherence (25, 26). Some studies have reported that only 44-46 % of the patients that are prescribed SLIT continue treatment after the first year (27, 28). Also, some patients experience disturbing local side effects during the first period of the treatment (29). So far, three allergens are available for SLIT therapy in Europe, grass, house dust mite and birch.

AIT is an expensive treatment in relation to the poor compliance in SLIT, high cost for the medical product, and is resource demanding with the need for medical supervision (SCIT) (30). Nevertheless, AIT is cost-effective by lowering the costs for pharmacotherapy and reducing the burden of uncontrolled disease in the society as a whole (12, 30, 31). Despite all positive effects AIT remains underused, likely due to safety concerns, issues with efficacy, and the long treatment regimens (13). Hence, there is a great incentive to find new ways to shorten the duration of AIT without losing the good effect or jeopardizing the safety.

2.3.3 New strategies for immunotherapy

2.3.3.1 Other administration routes

Alternative routes of allergen delivery have been explored to stimulate allergen uptake by antigen presenting cells and to avoid needle injections. Nasal administration by placing allergen on the nasal mucosa once weekly for 4 months has improved rhinitis symptoms, but induced bothersome local side effects for some patients (32). By placing allergen-adsorbed patches on the skin surface after the outer layers of the skin have been stripped by adhesive tape (epicutaneous administration) amelioration in seasonal rhinitis symptoms were obtained but also resulted in local and systemic side effects (33-35). Intradermal injections with low doses of grass allergen did not elicit any systemic reactions and late cutaneous responses were reduced (36). The authors speculate that the immune modulating effect despite the low allergen doses could be attributed to effective draining of allergen from the dermis to the lymphatics with aid from the antigen presenting Langerhans cells.

2.3.3.2 Adjuvants

Another approach to enhance the effect of immunotherapy is the use of adjuvants. Alum is the most widely used adjuvant for SCIT. The effect is mediated by a slow release of allergen, which prolongs the allergen presentation time (37). One side effect is the risk of developing alum contact allergy with itching noduli at the injection site. Possible bioaccumulation with long-term disease development have been discussed but have not been found clinically (37). Other adjuvants such as Toll-like receptor agonists and monophosphoryl lipid A (MPL) may further improve the outcome (38), but are still in the development phase.

2.3.3.3 Allergoids, peptide vaccines and recombinant allergens

In order to reduce the risk of allergic reactions the allergen can be modified. By processing the allergen chemically, allergoids can be produced that are less likely to induce reactions upon use, but still modulate the immune system. In Pollinex® Quattro, MPL and another adjuvant, microcrystalline tyrosine, is coupled to pollen allergen modified with glutaraldehyde and given as four pre-seasonal subcutaneous injections (39).

Another way of decreasing the potential for side-effects is to change the allergen protein structure by either creating recombinant proteins and/or cutting the proteins into peptides targeted to stimulate B- or T-cells (38, 40, 41). An open label study of a peptide derived from the Lolium perenne grass showed promising results with inhibited reaction in conjunctival provocation testing after 6 weeks of treatment. The use of MB32, a peptide from the IgE-binding site of grass allergen that is

fused to a carrier protein from Hepatitis B virus, is being developed to stimulate tolerogenic T-cell signals. An early trial showed that three subcutaneous injections could be performed without any early or late severe adverse allergic reactions and the nasal provocation test (NPT) in an allergen chamber showed reduced symptoms (42). A subsequent RDBPC trial that investigated MB32 in six injections during two years showed that the treatment reduced medication use and improved the scores at the Rhinoconjunctivitis Quality of Life Questionnaire (RQLQ), increased the allergen specific IgG levels without increase in IgE levels, but could not verify improvement in combined symptoms and medications scores (CSMS).

Although some positive and interesting results for the use of allergoids and peptide vaccines have been produced, it appears as these tentative approaches in AIT still have several hurdles to pass and a long way to go before reaching the clinic (40). With increasing insights in the mechanisms behind AIT many new types of modified allergens can be generated. Nonetheless, it is often difficult to translate promising results from early phase trials to robust therapies with improvement of symptoms in a real life setting (43) and a high cost for the vaccines for the development might limit the use.

2.4 Intralymphatic administration

2.4.1 Rationale

According to the "geographical concept of immune reactivity" an immune response can only be elicited with the antigen being inside secondary lymphoid organs such as the lymph nodes or the spleen (44, 45). The proposed mechanism behind tolerance development in AIT is that allergen has to be transported to lymph nodes where it can interact with T-cells and B-cells (45). A problem with SCIT is that the subcutaneous tissue contains low levels of antigen presenting cells which means that high doses of allergen must be used. There are also mast cells located in the tissue which confers a risk of allergic reactions.

By injecting an antigen directly into the lymph node, the immune system can be stimulated much more efficiently. The lymph nodes constitute an immunologically active system with high concentrations of antigen presenting cells such as dendritic cells (DCs) and B-cells. This increases the chances of interaction between the antigen and the specific T-cell. Lymph nodes contain low numbers of mast cells and basophils, which theoretically makes allergic hypersensitivity reactions less likely.

Biodistribution studies of intralymphatic and subcutaneous injections in mice (46) and humans (47) have shown that only a small fraction of subcutaneously injected proteins (e. g. allergens) reaches the lymph nodes. Most of the protein is drained to the liver where it is degraded, and a smaller proportion reaches the lymph nodes.

In one experiment the same dose of protein was injected in a lymph node and in the subcutis (47). After 25 hours the targeted lymph node and the adjacent nodes still contained a high concentration of protein. The draining lymph nodes close to the subcutaneous injection only had low levels of proteins 20 minutes and 25 hours after injection.

There are only few studies of ILIT in animals. One study showed that mice sensitized to cat fur were protected against anaphylaxis after intralymphatic allergen injections but not after subcutaneous ditto (46). Two trials showed that some dogs, but not all, with atopic dermatitis improved after receiving ILIT (48, 49). ILIT as primary prevention in horses induced favourable IgG antibody responses but the horses were not followed up to determine if they developed allergy (50, 51). Some mild local reactions were reported in the animal studies but no severe adverse events.

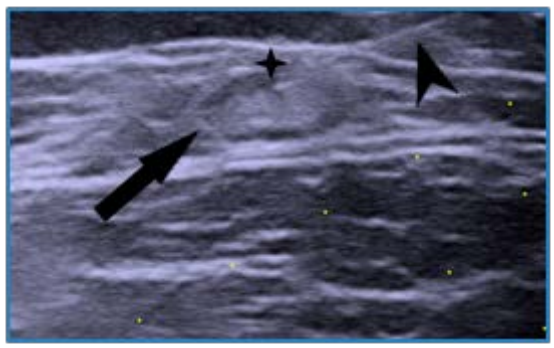


Figure 2. Ultrasonography picture of a lymph node, with a hypoechoic (dark) cortex and a central echogenic (light) hilum. Long arrow: Lymph node. Short arrow: Needle for injection. Asterix: Tip of the needle.

2.4.2 Human studies

The first clinical study in humans was an open randomized study where 58 patients received ILIT with grass allergen. After one and three years the ILIT-patients reported the same level of symptom relief as the patients that were allocated to SCIT (52). Other open studies include a trial of 7 patients treated with grass-ILIT that reported increased thresholds at NPT and skin prick test (SPT) and induced allergen specific plasmablast cells (53) and a trial of 10 patients also receiving grass-ILIT with reduced rhinitis total symptom scores (RTSS) and RQLQ scores (54). One recently published study from China treated 98 patients with AR induced by house dust mite, with allergen injection into cervical lymph nodes. Despite the sensitive location there were no serious local or systemic adverse reactions, and the authors found reduced RTSS and improved RQLQ scores (55). Another open

study used mixtures of house dust mite and animal dander allergens in up-dosing regimens, which gave good subjective symptom relief (56). In this study systemic and even anaphylactic reactions were reported, all other studies have demonstrated a good safety profile. In a recent open grass-ILIT study with a randomized booster injection the year after basic treatment, there were no significant improvement in CSMS but the specific IgG4 levels were increased (57).

Five randomized double-blind placebo-controlled (RDBPC) ILIT studies have been published. One trial evaluated ILIT with cat allergen. The recombinant allergen was modified with the allergen fused to an intracellular transporter molecule. This reduced the nasal reactivity upon allergen provocation and increased cat-dander specific Immunoglobulin G4 (IgG4) antibodies (58). Our research group showed that ILIT with birch or grass allergen improved symptoms at pollen season. It was a placebo controlled trial with 7 active patients (59), later expanded to 21 patients (60). The patients that had improved NPT also had increased allergen specific IgG4 affinities. A Danish grass-ILIT trial with 30 active patients could not verify any clinical effect (61). Treatment was given with a shorter dose interval (1-2 weeks instead of 4 weeks), which could have an impact on the immunological effect (62). ILIT with grass pollen in an up-dosing schedule in young adults showed improved symptoms scores at pollen season although not statistically significant in the small cohort of 7 active and 8 placebo patients (63). In a recently published study, patients were randomized 2:1 to receive ILIT with Japanese cedar pollen or placebo. The active group reduced reactivity at NPT and improved VAS. This trial remained blinded for three years and showed a sustained clinical effect for 1-2 years (123).

To summarize, nine out of ten human ILIT trials, not including the trials presented in this thesis, have shown results supporting the concept of ILIT. Two studies used dose-escalation protocols. Half of the studies used a study design with RDBPC measures. One of these included a follow up longer than the first season. Different extracts have been used regarding allergens and preparations, according to local relevance and availability of allergens.

2.5 Immunology in allergy

The immune system consists of biological processes and structures that have evolved to protect the body from disease and have to mount balanced responses to a wide range of pathogens. In AR, there is a pathologic response towards harmless antigens, that has many similarities with the normal Th2 response seen upon infection with extracellular parasites. This includes activation and differentiation of T-cells of Th2 type, IgE- producing B-cells, mast cells, basophils and eosinophils (64).

2.5.1 Inflammatory cells of the immune system

All cells of the immune system begin as bone marrow stem cells. These differentiate into lymphoid progenitor cells and later become lymphocytes (T-cells, B-cells, natural killer cells (NK) cells) or myeloid progenitor cells that differentiate into monocytes, granulocytes and erythrocytes.

2.5.1.1 Dendritic cells

When an antigen enters the body, the antigen presenting DC is the first cell that encounters the antigen. DCs originate from monocyte-like cells that migrate to the skin and mucosa. The main task of the DC is to take up and process pathogens, present antigens to T-cells and activate T-cells. Other antigen presenting cells in the skin and mucosa are monocytes and macrophages. DCs play a pivotal role at signalling the differentiation of T-cells (65). Immature DCs get activated either by cytokines (peptides used for cell signalling) from other leucocytes, or by recognizing foreign substances direct through pattern recognition. The activated DC take up small fragments of the antigen and travels via lymph vessels to a lymph node. Here the DC presents small peptides of the processed antigen to T-cells. There are two pathways for this presentation, major histocompatibility complex (MHC) I and MHC II. Extracellular, engulfed, substances are presented on MHC II, leading to activation of $CD4^+$ cells, T helper cells, which helps clearing extracellular infections. At an intracellular infection, substances produced inside the DCs are presented on MHC I molecules and signals an activation of $CD8^+$ T-cells, cytotoxic T-cells, that helps removing infected cells.

2.5.1.2 T-cells

Precursor thymocytes travel from the bone marrow to the thymus where the cells rearrange their T cell receptors and upon successful recognition of MHC/HLA class II or class I acquire their specific T-cell lineage markers, CD4 and CD8 respectively. After a proofreading in the thymic medullae, where autoreactive thymocytes are eliminated, naïve T cells leave the thymus and travel through the blood stream to secondary lymphoid organs, i.e., lymph nodes and spleen.

When a naïve T-cell in the lymph node encounters a peptide presented by the MHC molecule recognized by the unique TCR (T-cell receptor) , along with second co-stimulatory signals, the T-cell is activated.

When $CD4^+$ T-cells get activated, they proliferate, give activation signals to B-cells, and regulate other immune responses by secreting different mediators (66). Which type of mature T-cell the naïve T-cell develops into, depends on local cytokines and other factors. Cytokines like IL-2, TGF, IFN and IL-12 drive the development into Th1 cells. Other cytokines like IL-4 drive the development into Th2 cells. Thymic stromal lymphopoietin (TSLP) influences DCs to activate towards more

Th2 cells (67). Depending on which cytokines the $CD4^+$ T- cell in turn expresses, it is classified as a Th1, Th2 or Th17-cell important for defence in infections, regulatory T-cells (Treg) important for maintaining tolerance to self-antigens or follicular T-cells (fTh) that reside in lymphoid organs and activate B-cells (68, 69).

Th1 cells express the transcription factor T-bet and produce IFN-γ and TGF-β in response to intracellular pathogens and in non-allergic subjects towards allergens. IFN-γ stimulates cytotoxic T-cells in the defence from intracellular pathogens. IFN-γ also keep up the production of Th1 cells and suppresses the differentiation of T-cells into Th2 and Th17 cells (70). The allergen specific Th1 levels are increased in non-allergic subjects compared to allergic patients, and the increased fraction of Th1 can suppress Th2 pathways and the development of allergy (71). T-bet suppresses GATA-3 expression and Th2 differentiation (72).

Th2 cells express the transcription factor GATA-3 and produce interleukin (IL)-4, IL-5 and IL-13 that are involved in the clearance of extracellular pathogens. Th2 cells also mediate B-cells' switch in antibody production towards IgE and promote survival of eosinophils. In addition, GATA-3 suppresses production of cytokines that stimulate Th1 cells (72). IL-4 blocks the Th1 T-cell activation and maintains Th2 polarization (68).

By this, the Th1 and Th2 cells often act together in inflammatory reactions but when a pathway becomes dominant, as when the Th2 pathway gets exaggerated in AR, feedback loops of cytokines maintain the unbalance.

Th17 cells protects against bacterial and fungal infections and may have autoimmune properties, increase eosinophil recruitment but may also reduce neutrophil infiltration in asthma. Th22 independently express IL-22 and low amounts of IL-17, and play a role in atopic dermatitis (73).

Treg cells are paramount to maintain tolerance to self-antigens and commensal bacteria. In allergy, two types of Treg cells modulate the immune system. $CD4^+CD25^+$ innate Tregs that express the transcription factor FOXP3, and inducible IL-10 secreting type 1 Tregs. High levels of TGF-β, retinoic acid and short fatty acids promote Treg activity. Tregs suppress inflammation by production of IL-10, and by direct inhibition of cells e g DCs. The Treg function may be impaired among allergic patients (72). Induction of Treg cells is a key event in restoring the tolerance in allergy(73).

2.5.1.3 B-cells

B-cells secrete immunoglobulins that neutralizes extracellular microbes, and in allergy produce IgE antibodies. The B-cell is produced in the bone marrow as a pre-B-cell and then become a mature naïve B- cell without the influence of antigens.

The naïve B-cells express IgM and IgD on the surface. Each B-cell recognizes and reacts to only one specific antigen and the total B-cell population together can identify virtually all antigens the body might encounter. The naive B-cell circulates through the lymphatic system, prepared to meet its cognate antigen. B-cells that do not encounter its specific antigen die unactivated (67).

For activation of B-cells a signal from an antigen specific $CD4^+$ T-cell is needed to initiate cytokine production and gene expression. After the activation, the B-cell may differentiate to a long lived memory B-cell or proliferate to an immunoglobulin secreting plasmacell (74). The memory B-cells facilitate a quick response with a rapid build-up of antibody production at a subsequent encounter with the same antigen.

After activation, the B-cells undergo somatic hypermutation and by this rearrange the heavy chain DNA with maturation of the affinity of the antibodies, and with a change in Ig subclasses to IgA, IgG or IgE. The antibodies circulate to detect their antigen and mount humoral responses, except IgE that mostly binds to the high affinity receptor at the surface of mast cells and basophils. B-cells also function as antigen presenting cells and can secrete immunomodulatory cytokines with effects on the activation of T-cells and DCs (66, 67).

2.5.1.4 Mast cells and basophil granulocytes

Mast cells are granulocytes that are produced in the bone marrow and then travel to the skin and mucosa, where they can react upon antigens. IgE antibodies get attached to mast cells' FcεR1 and when IgE binds to its antigen, the receptor crosslinks with subsequent degranulation of the mast cells containing histamine and other inflammatory signals which attract e g eosinophils.

Basophils are one of the least common leucocytes in blood. They mature in the bone marrow and enter the circulation where they bind to IgE which crosslinks the receptors in a similar fashion as mast cells. The binding of IgE competes with IgG4. Basophils have long been thought to only have reactive properties but is now acknowledged to, under the influence of IL-3, secrete IL-4 that in turn favours Th2 responses (67, 75, 76).

2.5.2 Allergic type 1 reactions

Allergic rhinioconjunctivitis is an IgE mediated typ 1 reaction. There are also other types of allergic reactions; type II reactions with immunoglobulin binding and complement activation, type III reactions involving immune-complexes and type IV reactions mediated by T-cells rather than antibodies. They are not discussed further in this thesis.

In type I reactions, antigen presenting cells such as DCs and macrophages constantly scan the environment and engulf peptides, presenting them to T-cells. Sensitization occurs when an allergen is presented to a naïve allergen specific T-cell and this leads to activation of the T-cell which differentiate into a Th2 cell, which then undergoes clonal expansion. Th2-cells produce IL-4 and IL-13 that induce naïve B-cells to class switch antibody production towards IgE. Some B-cells become IgE^+ memory B-cells. Which signals that start the sensitization process are still not fully understood but they involve underlying genetic and environmental factors and Th2-cell activation under the presence of IL-4. When IgE is secreted it attaches to the high affinity receptor FcεR1 and sensitizes mast cells and basophil cells (77).

An immediate type 1 reaction occurs when basophils or mast cells are exposed to an allergen. The allergen binds to IgE, the receptor crosslinks and the cells degranulate and release anaphylactogenic mediators that cause the classical symptoms of AR (73). The cytokines from the granules can cause a subsequent late phase reaction when the Th2 cells produce cytokines like IL-4, IL-5, IL-9, IL-13, CCL5. These cytokines increase endothelial cell adhesion, migration of cells to the inflammatory site and increase activation of eosinophils. Th2 cytokines also stimulate B-cells to keep on with its production of IgE. Allergen specific Th2-cells that are reactivated expand clonally and migrate to the site of the allergen encounter, contributing to the late phase reaction. DCs and basophils both enhance the Th2 response (73).

2.5.3 Allergy immunotherapy

At AIT, much higher doses of allergen is used than seen during natural allergen exposures. Although intense research has been seeking for the answer to how this signals development of tolerance, a clear causal mechanism has not been established. A diverse set of immunological reactions have been observed at successful AIT, some connected, some seems to react in parallel.

2.5.3.1 Early desensitization

Very early protective events at AIT include the rapid desensitization of basophils which get less susceptible to degranulation (20). This happens within hours after the first allergen administration in AIT. One proposed mechanism is related to the rapid upregulation of histamine receptors that counteracts the crosslinking of FcεR1, preventing its degranulation (78). The inactivation of basophils and mast cells have subsequent effects on T-cells and DCs.

2.5.3.2 T-cells

AIT also induces marked effects on allergen specific T-cells. As discussed previously, in allergic disease, the T-cell balance is skewed towards Th2 activation. At AIT, the T-cell balance is restored towards increased levels of T helper (Th) type

1 and regulatory T (Treg) cells and reduced amounts of IL-4 secreting Th2 cells. The mechanism by which Tregs are activated is not fully elucidated. Tregs have various allergen specific immune suppressive functions (73). There is a strong correlation between the number of Tregs induced and clinical improvement after AIT (20). Tregs suppress DCs by contact inhibition which suppresses the generation of the effector T-cells Th1, Th2 and Th17. In addition, Tregs produce IL-10 that suppress IgE production of B-cells and enhance a class switch to blocking IgG antibodies and in particular IgG4. Allergen specific IgG4 compete with IgE in binding to the receptors at basophils and mast cells and prevents activation and degranulation. Tregs also secret TGF-β which maintain enhanced Treg production, and suppresses basophils' and mast cells inflammatory functions (45).

As signs of T-cell adaptations, one ILIT study presented decreased levels of Th2 cells and increased Treg cells and IL-10 levels in blood during ILIT, but the levels returned to normal after the treatment (54). An ILIT trial with a modified cat allergen showed increased activation of T-cells shortly after treatment but unresponsive T-cells to allergen 1 year after treatment (79). An ILIT study that investigated a shorter dose interval could not detect any clinical effect and the immunological investigations showed reduced levels of interferon-γ (IFN- γ) (61), which could be a sign of unfavorable T-cell responses, as an immunological explanations to the result.

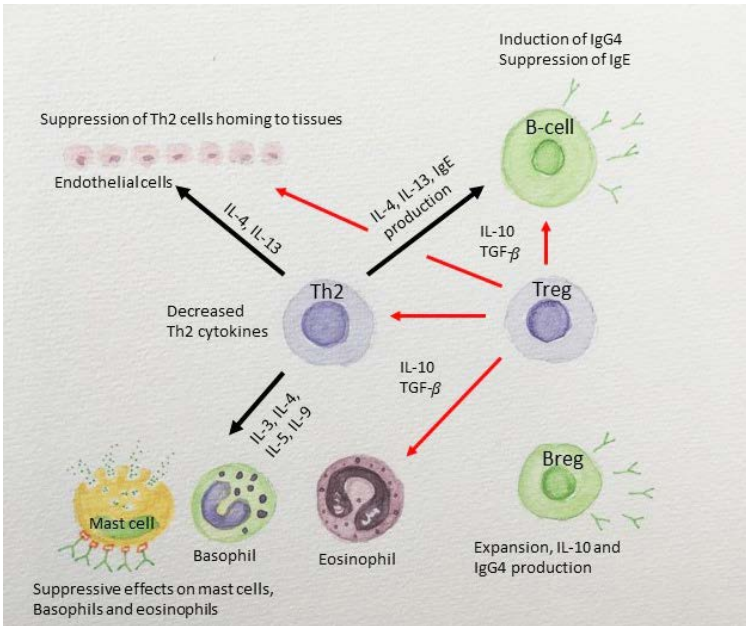


Figure 3. Overview of Treg and Breg (Br1) functions. Red arrows indicate suppression of allergic inflammation. Adapted from Akdis, World Allergy Organization Journal, Volume 8, 2015, 17.

2.5.3.3 B-cells

Early in AIT there is often a rise in allergen specific IgE levels (Fig. 4). The levels are then gradually decreased during the treatment, but the symptom improving effect is not dependent on this decrease, that occurs relatively late during treatment. Later tolerogenic events in AIT include the induction of regulatory B-cells that secrete IL-10 which suppresses $CD4^+$ effector inflammatory functions and promotes IgG4 production in B-cells (20, 73). A naturally occurring tolerance induction is observed among bee-keepers and cat owners. Allergen specific Breg cells that produce IL-10 and a subsequent increase in allergen specific IgG4 has been found among bee-keepers that have developed a natural tolerance to bee venom (80).

Some previous ILIT trials have reported increased levels of allergen specific IgG4 levels (56, 58) with associated symptom improvement. In one study the IgG4 levels were unchanged, but an increased affinity was found in the subgroup that exhibited improved response to NPT (60). One ILIT trial could not verify any increased IgG4 production but as an indirect sign of B-cell modulation, an increase in plasmablasts not producing IgE was seen(53).

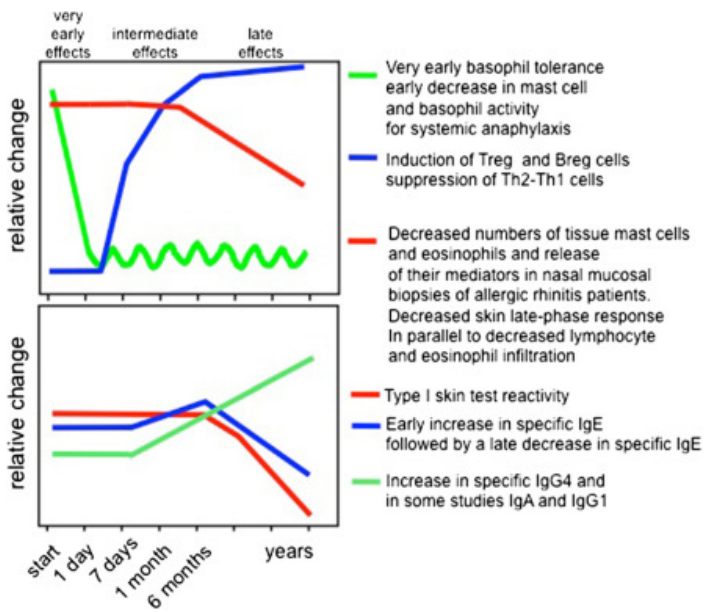


Figure 4. The timing of tolerogenic events after allergy immunotherapy. From Akdis, World Allergy Organization Journal, Volume 8, 2015, 17.

2.5.4 Immunologic mechanisms in ILIT

Immune responses are initiated in secondary lymphoid organs, it therefore seems to be a good chance for efficient stimulation when the allergen is injected into the nodes. They contain high numbers of T-cells and B-ells that can interact with the allergen. Many studies have indicated similar immunological mechanisms in ILIT as in other types of AIT, such as increase in IL-10 and allergen specific IgG4, induction of non-IgE producing plasmablasts, increase in Treg cells and a long-term T-cell unresponsiveness to allergen. One ILIT study measured the basophil reactivity but could not detect any change, despite improved symptoms after NPT and decreased SPT responses(53). It is possible that the mechanisms in ILIT are partly different from other AIT, since the first step of allergen exposure at the mucosa or close to the skin, is circumvented.

3 MATERIALS AND METHODS

3.1 Study design

The overall study design in paper I-III was a series of RDBPC trials where the study subjects were randomized to active ILIT or placebo ILIT in parallel groups. Paper IV was an open follow-up study 5-6 years after the RDBPC trial in paper II. (Fig 5-7).

The patients were recruited at our study centers; Karolinska University Hospital, Skåne University hospital in Malmö and Lund, and Södra Älvsborg Hospital in Borås. In study I and II randomization was achieved with opaque envelopes. A nurse not connected to the study drew one envelope for each patient and prepared the medical product according to the study arm assignment in the envelope (active or placebo). In study III, a computer-generated randomization plan was used.

3.1.1 Patients

General indications for conventional AIT were followed. Inclusion criteria were a history of moderate to severe AR according to ARIA guidelines at the pollen season, positive SPT and allergen specific IgE levels >0.3 kU/L (1). Exclusion criteria were severe atopic dermatitis, uncontrolled perennial asthma, symptomatic sensitization to house dust mite or furry animals with daily exposure, use of beta blockers or ACE inhibitors as antihypertensive medications, pregnancy or nursing, wish for pregnancy, known autoimmune or collagen disease, previous immunotherapy, obesity with BMI >30 due to potential difficulties visualizing lymph nodes with ultrasound, other significant diseases or withdrawn informed consent.

Paper I: 30 patients aged 16 to 42 with mild asthma and AR towards birch or grass pollen.

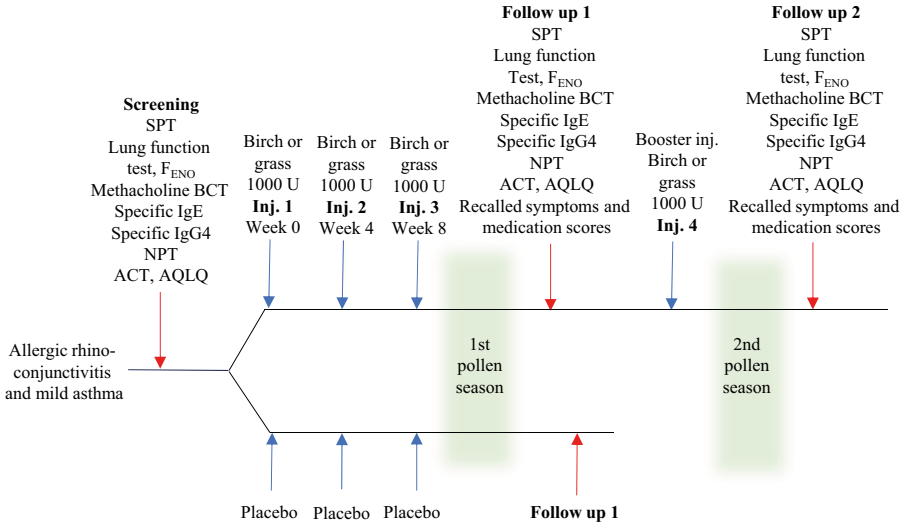
Paper II: 60 patients aged 18-55 with moderate to severe AR towards birch and grass pollen.

Paper III: ILIT after SCIT- 10 000: 29 patients aged 18-55 that had recently completed SCIT for grass induced AR. **ILIT de novo- 3000:** 39 patients aged 18-55 with moderate to severe AR towards grass without previous AIT treatment.

Paper IV: 20 patients aged 18-55 with moderate to severe AR, previously treated with active birch and grass ILIT within a RDBPC trial 5-6 years previously. 14 patients with moderate to severe AR towards birch and grass, without previous AIT.

All studies were approved by the Ethical Review board in Stockholm and/or Lund and the Swedish Medical Products Agency, conducted according to good clinical practice guidelines and registered at ClinicalTrials.gov.

Paper I- ILIT young adults with mild asthma



Paper II- ILIT with two concomitant allergens

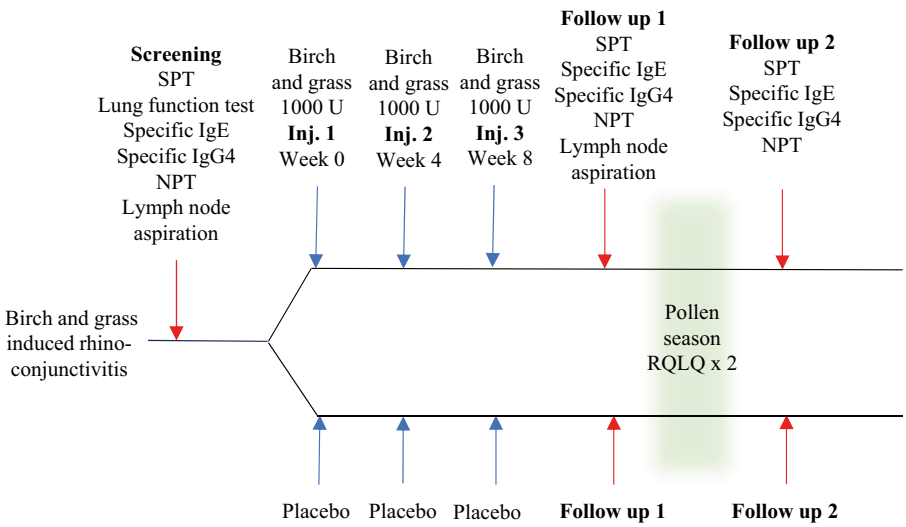
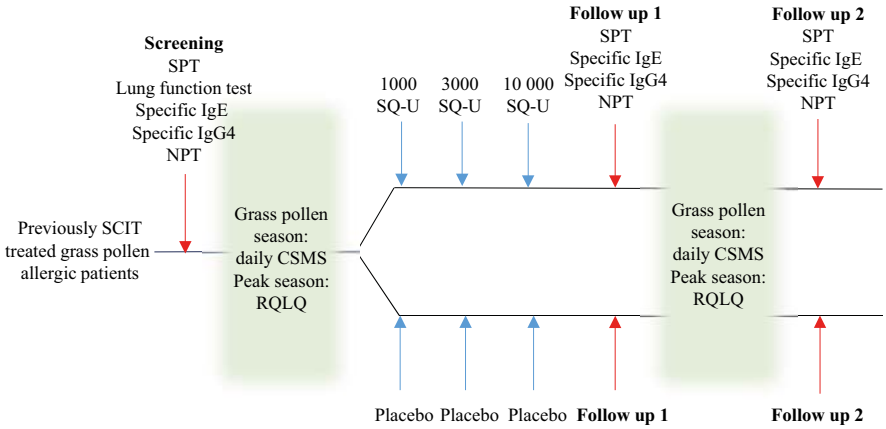


Figure 5. Study outline in paper I and paper II. SPT= skin prick test, F_{ENO}= fraction of exhaled nitric oxide, BCT= bronchial challenge test, NPT= nasal provocation test, ACT= asthma control test, AQLQ= Asthma Quality of Life Questionnaire, RQLQ= Rhinitis Quality of Life Questionnaire.

Paper III- ILIT after SCIT- 10 000



Paper III- ILIT de novo- 3000

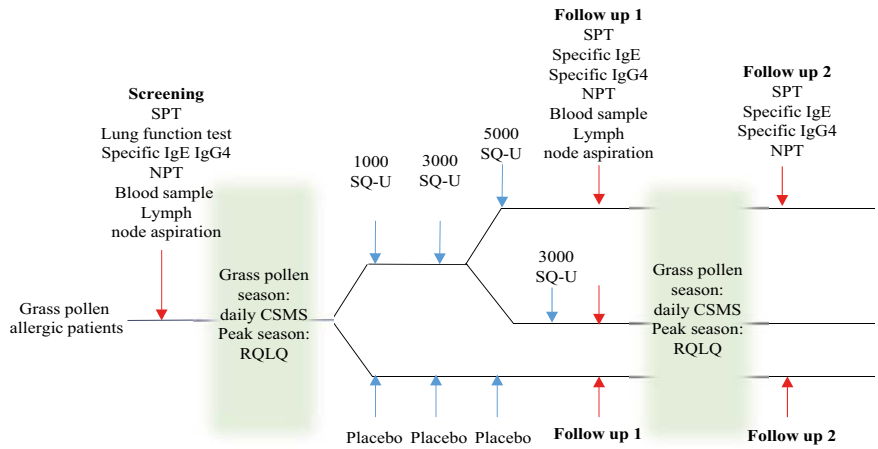


Figure 6. Study outline in paper III. SCIT= subcutaneous immunotherapy, SPT= skin prick test, NPT= nasal provocation test, CSMS= combined symptoms and medication scores, RQLQ= Rhinitis Quality of Life Questionnaire.

Paper IV- ILIT 5-year follow-up

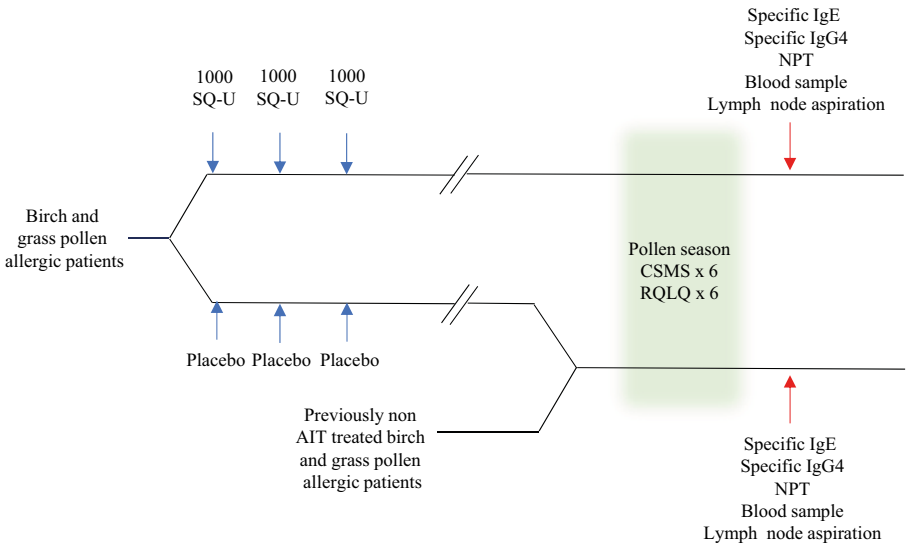


Figure 7. Study outline in paper IV. NPT= nasal provocation test, CSMS= combined symptoms and medication scores, RQLQ= Rhinitis Quality of Life Questionnaire.

3.1.2 Treatment protocols

3.1.2.1 Study I- ILIT in asthmatic young adults

30 patients received randomized treatment during 2012-2016 at Karolinska University Hospital. The primary outcome measure was reduction in NPT reactivity. Secondary outcomes were safety, AR and asthma symptoms and medication use during pollen season, allergen specific IgE, IgG and IgG4 levels, SPT, reaction at a methacholine hyper responsiveness test, fraction of exhaled nitric oxide (F_{ENO}), asthma control test (ACT) scores and Asthma Quality of Life Questionnaire (AQLQ) scores. Active product was ALK Alutard® birch or grass with the dose 1000 SQ-U. Three injections were given with 3-4 weeks interval. Placebo patients ended their participation after the first year. Patients that had received active treatment returned the next year for an open pre-seasonal 1000 SQ-U injection 1-4 months before the pollen season. In this open part of the study the same readout parameters were repeated.

3.1.2.2 Study II- Intralymphatic immunotherapy with two concomitant allergens- a RDBPC trial

60 patients were recruited during 2012-2015 at Karolinska and Skåne University hospitals. Active treatment was ALK Alutard® birch and grass with the dose 1000 SQ-U. Three injections were given with 3-4 weeks interval. Both birch- and grass allergens were given at the same visit, with 30 minutes of observation between the injections. The primary outcome measure was the symptoms at NPT with grass allergen. Secondary outcomes were safety, allergen specific IgE and IgG4, SPT, RQLQ, use of pharmacological treatment at pollen season, levels of T-cells and changes in T-cell activation in blood and lymph nodes.

3.1.2.3 Paper III- High dose grass pollen intralymphatic immunotherapy: two RDBPC trials question the benefit of dose increases

In this paper, two trials investigated ILIT in up-dosing schedules, ILIT after SCIT-10 000 and ILIT de novo- 3000.

ILIT after SCIT- 1000 was a pilot study aimed at investigating the safety of a novel up-dosing regimen. The aim was to investigate if ILIT, with a higher allergen concentration than previously used, induced further amelioration of the allergy symptoms in patients already treated with SCIT. 29 patients were included during 2015-2016 in three participating study centers in Sweden. Inclusion criteria were age 18-55 and a recent completion (within 20 months) of a full 3-year SCIT-program with amelioration of symptoms without reaching full symptom relief. The medical product and dose interval was the same as in previous studies but the doses were increased: Treatment 1: 1000 SQ-U. Treatment 2: 3000 SQ-U. Treatment 3: 5000 SQ-U + 5000 SQ-U with 60 minutes' observation in between the injections. The primary outcome parameter was the CSMS during pollen season. Secondary outcome measures were safety, allergen specific IgE-and IgG4 levels, SPT, NPT, and RQLQ.

ILIT de novo- 3000 included 39 patients aged 18-55 with moderate to severe AR towards grass pollen that had not undergone previous AIT. This trial was performed the year after ILIT after SCIT- 10 000, at Karolinska and Skåne University Hospitals. The regimen for treatment aimed at the same as in ILIT after SCIT-10 000. However, due to adverse reactions, the protocol was changed to: Treatment 1: 1000 SQ-U. Treatment 2: 3000 SQ-U. Treatment 3: 3000 SQ-U. The outcome measures were the same as in ILIT after SCIT- 10 000 and, in addition, distribution and activation of T-cells and DCs in blood and lymph nodes.

3.1.2.4 Paper IV- A five-year open follow up of a randomized double-blind placebo-controlled trial of intralymphatic immunotherapy for birch and grass, reveals remaining beneficial effects

20 patients that had 5-6 years previously participated in the RDBPC trial described in paper II, treated with active birch and grass ILIT, were compared to 14 patients with moderate to severe AR towards birch and grass, without previous AIT. In the non-AIT treated control group, 8 patients had previously participated in the RDBPC trial. The primary outcome parameter was the birch and grass NPT. Secondary outcome measures were CSMS during pollen season, allergen specific IgE-and IgG4 levels, NPT, RQLQ, basophil activation test and distribution and activation of cells in lymph node and blood.

3.1.3 Intralymphatic injections

For the intralymphatic injections the lymph nodes in the groin were used. Here the lymph nodes are located shallow in the subcutaneous tissue. The injections were performed with aseptic technique and ultrasound guidance. To facilitate identification of the lymph node the ultrasound picture was saved after injection. The same lymph node was then targeted with the same allergen at all injections. In study II, grass pollen was given in the left groin and birch pollen in the right groin.

3.2 Evaluation of clinical improvement

3.2.1 Global assessment of symptom relief

Scoring on a visual analogue scale (VAS) is a fast and easy way to assess the overall symptomatic impact of AR for patients and researchers. Patients usually grade their symptoms on a continuous scale ranging from 0: “no symptoms” to 10: “highest level of symptoms”. This psychometric response scale has been used in several conditions. VAS is also validated for AR (82). The scale can be used in a comparative fashion in order to evaluate a treatment. In that case the extreme limits at the scale are labelled with 0: “no relief” and 10: “complete relief” (83). An advantage with the comparative scale is that the magnitude of the response does not depend on the severity of the initial condition. We used this type of comparative VAS in our studies. Disadvantages of the relief scale is that it creates the impression that all patients start at the same level of disease severity which may mask differences in outcome between patients. Also, patients need to recall their initial symptoms before they can assess their relief, which affects the reliability (83).

3.2.2 NPT

To evaluate the rhinitis symptoms at allergen exposure, a nasal provocation (NPT) test can be conducted. It can be done by titrating increasing concentrations of allergen to determine the threshold that induces symptoms (84-86). It can also be performed by evaluating symptoms after only one dose (87, 88). To complement the subjective experience of responsiveness to allergen, objective measurements of the nasal air flow can be achieved with rhinomanometry or peak nasal inspiratory flow (PNIF) (89).

An advantage of a provocation test is that the allergy symptoms are measured after the same allergen exposure for all patients and without the influence of symptom-ameliorating pharmacotherapy. A weakness is that NPT does not fully represent the real-life seasonal pollen exposure (90, 91). NPT is recommended as a surrogate end point in proof-of-concept studies and novel AIT approaches (91).

In our trials, the patients were challenged with 1000 SQ-U of ALK Aquagen® timothy or birch in each nostril. The patients scored rhinitis and conjunctivitis symptoms 0-3 at 0, 5, 15 and 30 minutes.

3.2.3 Asthma

Asthma symptoms in study I were evaluated with the ACT before treatment and after the end of the pollen season (92). The score ranges 0-25 where 19 points or below indicates risk of uncontrolled asthma. A lung function test with measurement of FEV1 and forced vital capacity was performed according to international standardization (93). Bronchial hyperresponsiveness to a methacholine challenge was assessed where the dose of methacholine that caused 20% reduction of FEV1 (PD_{20}) was calculated (94).

3.2.4 Quality of Life

Quality of life (QoL) is an important parameter when assessing disease burden of most conditions studied. There are several generic QoL questionnaires available as for example Medical Outcome Study 36-item short form (SF-36) (95). When studying changes within a medical condition a disease-specific questionnaire is often more sensitive (96). For allergy-related QoL, the Juniper Quality of Life Questionnaire (RQLQ) (97, 98) is widely used and recommended (91).

In paper II-IV, the score was calculated as the average of 28 questions, each ranging 0-6 (resulting in maximum RQLQ score 6 points) and the minimal clinically important improvement is 0.5 point (98). For asthma related QoL scoring in study I, the AQLQ score was used (92) with a similar 0.5 point level of minimal clinically important difference.

3.2.5 Daily combined symptoms and medication scores

A symptom- and medications diary is considered to be the most effective way to evaluate seasonal allergic symptoms (91), but is resource-demanding for study participants. There is a well-defined terminology for the symptoms scores (SS) in the eyes (ocular itching/grittiness/redness and ocular tearing) and for the symptoms in the nose (nasal itching, sneezing, rhinorrhoea and nasal obstruction). These symptoms are scored 0-3 every day during the pollen season. This allows tracking of the different symptoms and interpretation of the symptoms in relation to different pollen levels during the season. Daily scoring prevents recall bias.

The consumption of rescue medications during pollen season is also recorded as medication score (MS). Numerous different ways to grade the use of different medications have been applied, for example use of antihistamine gives 1 point and the use of nasal steroid gives 2 points, etc. (91). The term rescue medication is often used for the symptom ameliorating medication that is used by the patients at natural allergen exposure (e.g. pollen season) during the study period. This has nothing to do with medications used as treatment for allergic side reactions provoked by the immunotherapy treatment.

The SS and MS can be used separately or, preferably, weighed together, since the use of pharmacological treatment has an impact on the symptoms. Recently, a European Academy of Allergy and Clinical Immunology (EAACI) task force recommended a simplified and standardized scoring system (91). In paper III we used this scoring system and all patients were instructed to use their medications stepwise, as needed, following the ARIA guidelines (99). The registrations were performed at baseline during the pollen season before the treatment and at the pollen season after the treatment.

3.2.6 Modified symptoms and medication scores

In paper I, modified SSs and MSs were assessed before treatment and after the first pollen season. The scores were calculated taking into account the frequency: daily (4 points); every second day (3 points); 1 to 3 days per week (2 points); occasionally (1 point); never (0 points), for the following symptoms: blocked nose, rhinorrhea, fatigue, sneezing, and asthma symptoms, and for the following medications used: local and systemic antihistamines, nasal steroids, asthma medication, and eye drops. A maximum score of 20 points for symptoms and 16 points for medication could be obtained.

In paper II, the use of antihistamine tablets, ocular antihistamines drops, intranasal steroid spray, corticosteroid tablets, β2 inhalation spray and corticosteroid inhalation spray were assessed after the first pollen season as reduced, unchanged or increased.

In paper IV the same CSMS scores were used as in paper III, but repeated at six occasions during the birch and grass pollen season.

3.3 Immunological methods

3.3.1 Commercially available immunological tests

AR is an IgE-mediated disease. The diagnose relies on a typical history of rhino-conjunctivitis symptoms at allergen exposure and either positive SPT or elevated serum-levels of allergen specific IgE antibodies. IgE can be measured with ImmunoCAP™ (Thermo Fisher Scientific, Uppsala, Sweden) at most hospital laboratories. The technology relies on high and specific binding capacity to a solid phase. The test is designed as a sandwich immunoassay. Allergen is covalently coupled to the solid phase, which then reacts with the specific IgE in the patient serum sample. Non-specific IgE is washed away and antibodies against IgE are added to form a complex. Unbound anti-IgE is washed away in a second step and the bound complex is incubated with a developing agent. The agent is fluorescent after the reaction has stopped and the intensity of the fluorescence correlates to the IgE level in the sample (100).

Elevated serum-levels of allergen specific IgG4 antibodies are often associated with allergen exposure and/or tolerance induction and can be measured to monitor the immune response and/or compliance in AIT (20). This test is also available as a fluoroenzyme immunoassay at the hospital laboratories, based on the ImmunoCAP™ technology.

3.3.2 SPT

Skin prick test (SPT) is a traditional method for demonstrating an allergic reaction to a known allergen by provoking a small, immediate, allergic reaction in the skin. A panel of different allergen-containing drops is placed on the volar side of the forearm. A small lancet introduces the allergen shallowly into the skin (101). A positive control with histamine chloride in a concentration of 10 mg/ml and a negative control with saline buffer is used to confirm that the test is working. A wheal reaction of ≥3 mm after 15 minutes is usually considered positive.

3.3.3 Flow cytometry

In paper II-IV, T-cell characteristics were determined with flow cytometry. In paper III, activation of DCs in blood and lymph nodes were also investigated. In paper IV we focused on T-cells and basophil activation.

With ultrasound guidance, the lymph node was aseptically punctured with a 22-gauge needle, aiming at the cortex/paracortex area. Lymph node aspirates were suspended in sterile PBS (Gibco™, Life Technologies, Uppsala, Sweden). When sampling blood, tubes containing a buffered trisodium citrate solution were used. Peripheral blood mononuclear cells (PBMC) were separated from whole blood

with ficoll density centrifugation (GE Healthcare, Buckinghamshire, UK). Flow cytometry was performed within 12 hours, with an LRS Fortessa (BD Biosciences) and the data were processed using FlowJo software© version 10.6.2 (Tree Star, Inc., Ashland, USA).

Flow cytometry can measure several physical and chemical properties of single cells. It can be used in order to study the phenotype and understand the function of cell subsets. Suspended cells pass through a laser beam and individual cells scatter the light differently (102). This enable measurements of the cell, regarding the cells' size (Forward scatter, FSC) and granularity (Side scatter, SSC). The data are plotted in a two-dimensional dot-plot that can describe the relation between two different characterizations at a time. Specific areas of interest in the dot plot can be separated and monitored more closely, a process called "gating". For example, the correlation between FSC-Height and FSC-Area allows gating of single cells and discard doublet cells (Fig. 8A). In the same way dead cells and debris that have low FSC-height can be gated away. The expression of intra- or extracellular proteins can be measured by adding a fluorochrome-conjugated antibody that binds to the antigen on the cells. The number of cells that are positively labelled can be measured, as well as the median fluorescence intensity (MFI). For example, T-cells can be labelled and gated based on high expression of CD3 (Fig. 8B). These $CD3^+$ T-cells can subsequently be divided into $CD4^+$ and $CD8^+$ T-cells per expression of the differentiation cell surface protein CD4 (Fig. 8C).

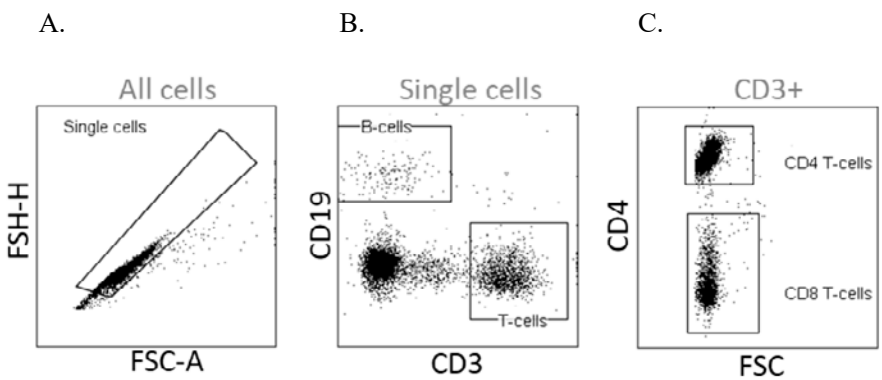


Figure 8. Flow cytometry. A. FSC-Height and FSC-Area. **B**. Expression of CD19 and CD3. **C.** Expression of CD4.

To characterize the cells in our studies, we used CD19 (B-cells) and CD3 (T-cells), CD4 (T helper cells) and CD8 (cytotoxic T-cells). To further determine the T-cells we used $CCR7^+CD45RA^-$ (central memory T-cell), $CCR7^+CD45RA^+$ (naïve T-cell),

$CCR7^-$ (effector memory T-cell), CCR5 (Th1) and CRTH (Th2). We also used $CD45^+$, SSC^{++} and $CD11C^+$ (DCs), expression of CD80, CD86 and CD141 (activation of the DCs) and specific basophil markers mentioned below. To exclude monocytes in the analysis of blood we used CD14. For determination of positivity we used internal controls and fluorescence minus one controls.

3.3.3.1 Characterization of basophil cells

In paper IV, we investigated the activation of basophil granulocytes. These cells contain preformed granules, or vesicles, containing e g histamine. Positively and negatively bound molecules help forming the vesicles. When allergens crosslink IgE attached on the cell surface, the basophil cell degranulates by fusing the vesicle membrane with the cell membrane. The expression of the vesicular membrane marker CD63 (103) and the positively charged marker for vesicular content avidin (104) were measured with flow cytometry. The blood was stimulated with 100 SQ-U of grass allergen (0.2 mL of 500 SQ-U/mL ALK Aquagen®). Before flow cytometry analyses, the samples were incubated with antibodies enabling detection of basophil cells by their expression of HLA-DR and IgE. Other markers in the panel included CD45 (for setting the gate at the leucocyte detection area), CD63 and avidin (markers for activation), FcεR1 (IgE receptor) and membrane bound IgE (105).

3.4 Statistical analyses

For the statistical analysis GraphPad Prism 6.01 software was used (San Diego, CA, USA). Statistical significance was set at $p<0.05$. NPT and other parameters measured repeatedly were analyzed with Friedmans test followed by Dunn's multiple comparisons test when the data was not normally distributed or RM one-way ANOVA if normally distributed. CSMS and other paired observations were analyzed with Wilcoxon match-pairs signed rank tests or paired t-test. VAS and other unpaired observations in the active versus the placebo or non AIT-treated group were analyzed with Mann-Whitney tests to compare ranks or un-paired t-test. Unpaired observations of DC activation were analyzed with Kruskal-Wallis test. The proportion of patients that changed medications were analyzed with Chi-square tests. Power calculations were performed in all studies with a 2-sample t-test for the different primary outcome variables, and by comparing two paired means in paper IV. In paper III a generalized additive model (GAM) was used in a time-series analysis to evaluate the relative risk (RR) for allergy symptoms in relation to the pollen levels. These data were analyzed with the software R (Version R 3.3.3 GUI 1.69 Mavericks build, Vienna, Austria).

In the trials, all the patients that received one or more ILIT injections were included in the safety analysis. All the patients that completed the treatment and

follow up protocol were included in a modified intention-to treat (mITT) efficacy analysis. By this approach, patients that had missing data for any outcome were excluded from that particular analysis, but remained in the analysis of all the other parameters in order not to lose power (106). In addition, any patients that were found ineligible after randomization or did not complete the treatment were excluded from the analysis. The use of mITT analysis have been overrepresented at for-profit sponsorship trials and is described to confer a risk of arbitrary post-randomization exclusions (107). However, the studies included in this thesis had no relevant conflicts of interests and all exclusions were justified and described in the CONSORT flow diagrams (108).

4 RESULTS

4.1 Paper I- Intralymphatic immunotherapy in pollen-allergic young adults with rhinoconjunctivitis and mild asthma: A randomized trial

30 patients with mild asthma and moderate to severe AR induced by birch or grass pollen, were randomized double-blind to three intralymphatic injections of the corresponding allergen, or placebo. The doses were 1000 SQ-U at each injection, with one month-intervals. The active group returned for an open booster injection of 1000 SQ-U the second year. 14 active patients with median age 19.5 years and 12 placebo treated patients with median age 18 remained for analysis. In the active group, 11 patients had birch pollen induced AR and 3 patients had grass pollen triggered disease. In the placebo group 8 patients had birch pollen allergy and 4 patients grass pollen induced symptoms.

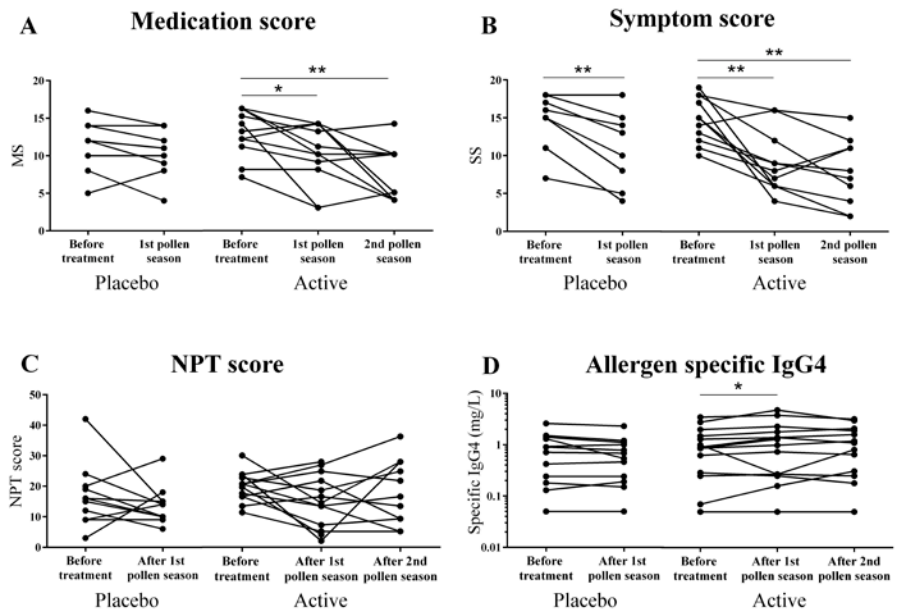


Figure. 9. A. The MS were reduced in the active group but not in the placebo group. **B.** The SS improved in both the active and in the placebo group. **A-B.** (Active group n=11 at 1st pollen season, n= 10 at 2nd pollen season. Placebo group n= 9) **C.** The NPT score did not change in the active (n=12), or in the placebo group (n=11). **D.** Allergen specific IgG4 antibodies (depending on the relevant allergen, birch or grass) were increased in the active (n=14 after 1st pollen season, n=13 after 2nd pollen season) but not in the placebo group (n=12). *p<0.05, **p<0.01.

Limited local reactions at the injection site were common. One patient had an urticarial rash in the forehead 20 minutes after the booster injection. This was successfully treated with corticosteroids and antihistamines and the patient could be discharged from the hospital after 4 hours of observation. One patient reported increased asthma symptoms during the day after the last injection. One patient got salivary glands swelling and cough 2 months after the booster injection and was diagnosed with sarcoidosis. Ultrasound examination of the lymph nodes that had received treatment did not reveal any swelling or granular appearance. The manifestations improved after systemic steroids and Methotrexate. No causal relationship with ILIT has been established.

The MS reflected the frequency of applications of the medicines and were reduced in the active group during the first pollen season after treatment. Placebo treatment did not change the need for rescue medications. After the open label booster ILIT injection year two, the MSs were further reduced (Fig. 9A). The recalled symptom scores (SS) were calculated considering the frequency and severity of the symptoms. SS improved in both the active and the placebo group after treatment. The score of the NPT was the sum of the symptom scores after the challenge, which did not change after treatment. The allergen specific IgG4 antibodies were increased 6-9 months after active ILIT, but returned to baseline 2 years after treatment, despite the booster injection. The placebo group did not change. (Fig. 9D.) Allergen specific IgG levels were also increased the year after active treatment but returned to baseline year two. There were no detectable changes in the allergen specific IgE levels. The clinical parameters improvement on VAS, ACT, AQLQ, response to bronchial methacholine challenge and measurements of FEV_1 and F_{ENO} did not change, neither in comparisons within the groups nor in comparisons between the active and the placebo group.

4.2 Paper II- Intralymphatic immunotherapy with 2 concomitant allergens, birch and grass: A randomized, double-blind, placebo-controlled trial

60 polysensitized patients,18-55 years old with birch and grass pollen induced moderate to severe AR were randomized double blind to three intralymphatic injections of birch and grass allergen, or three injections of placebo. The treatments were given with one month-intervals and the doses were 1000 SQ-U at each injection. 27 placebo patients and 24 active patients remained for analysis.

The adverse events were mostly mild. In total 12 events of local reactions >5cm in size, were reported in the active group. One patient had a 5 cm local reaction combined with a genital herpes zoster reactivation and left the study after that event. One patient was treated with antihistamines and corticosteroid tablets. Two active patients had mild rhinoconjunctivitis symptoms the day after the first injection. No other relevant systemic reactions were reported and no Adrenaline was used.

The grass allergen reactivity following NPT was reduced by 28% in the active group 6-9 months after the treatment, compared to the baseline. The placebo group exhibited no reduction (Fig. 10).

The RQLQ scores during the birch pollen season showed a trend for improvement in the active group with an average 1.0-point reduction of the symptom scores in the active group compared to placebo (Fig.10). Some domains displayed significant differences; mean nasal symptom scores were 1.1 points lower in the active group than in the placebo group, non-nose/eye symptom scores were 1.0 points lower and emotional symptoms scores were 0.8 points lower than in the placebo group.

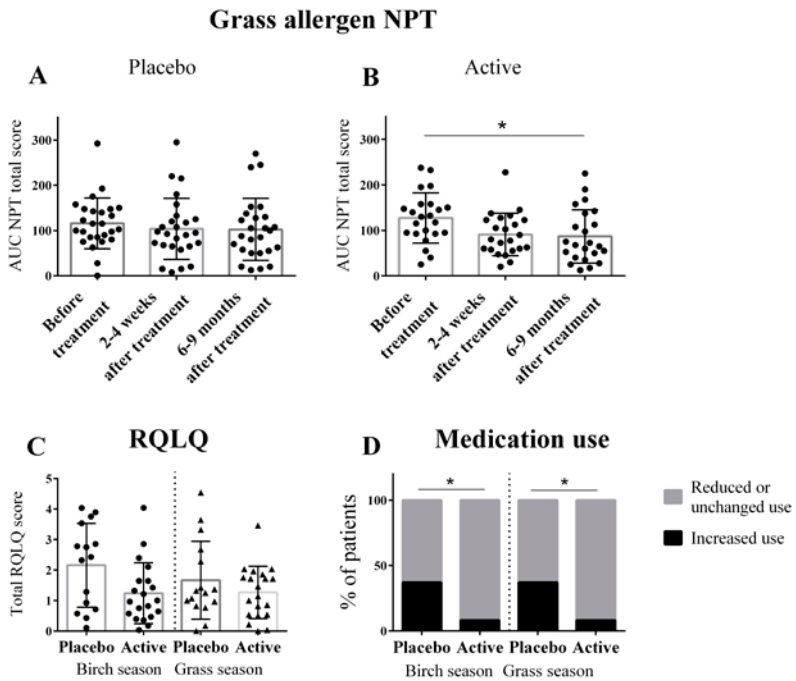


Figure 10. A-B. The grass allergen NPT showed 28% reduced reactivity in the active group (n= 23) 6-9 months after treatment while the placebo group did not change (n=26). C. The total RQLQ scores reported were lower in the active group (n=20) compared to the placebo group (n=15) at the birch and grass pollen season after treatment, but the difference did not reach significance. **D.** More patients in the active group (n=24) had a reduced or unchanged use of antihistamines and/or nasal steroids than in the placebo group (n=27). *p<0.05. **A-C** Horizontal lines at mean and SD.

The medication use was more reduced in the active group than in the placebo group after treatment. After the pollen season, 92% of the active patients reported less or unchanged use of antihistamine tablets and/or nasal steroid, whereas 63 % of the patients

in the placebo group reported unchanged or reduced use. Treatment effects measured as improvement on VAS, scored after the pollen season, did not show any changes.

The grass specific IgG4 as well as IgE antibodies in sera were increased in the active group after ILIT, whereas the birch specific IgG4 and IgE levels remained unchanged (Fig. 12). Flow cytometry analysis of lymph node material revealed an increased amount of CD4+ and $CD8^+$ memory T-cells after ILIT. For both cell populations, the effector memory T-cells increased more than the central memory T-cells. In blood, the Th1 cells (determined as $CCR5^+$ $CD4^+$ T-cells) and Tregs (determined as $CD25^{++}$ $CD4^+$ EM T-cells) increased (Fig. 11).

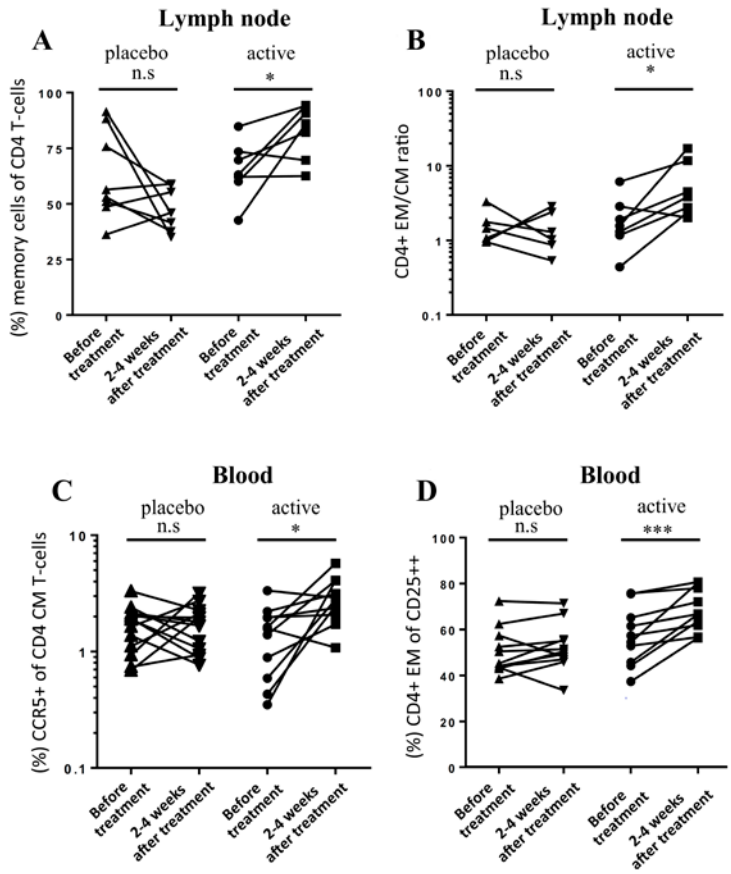


Figure 11. A. The amount of $CD4^+$ memory T-cells increased in the ILIT-treated lymph nodes (n=7) but remained unchanged after placebo treatment (n= 8). **B.** The effector memory T-cells increased more than the central memory T-cells, in the active group (n=7). There were no changes of the proportions in the placebo treated lymph nodes (n=6) **C.** The Th1 cells increased in blood after active ILIT (n=11) but not after placebo (n=12). **D**. The Treg cells increased in blood after active treatment (n=10) but were unchanged in the placebo group (n=10). *$p<0.05$. ***$p<0.001$.

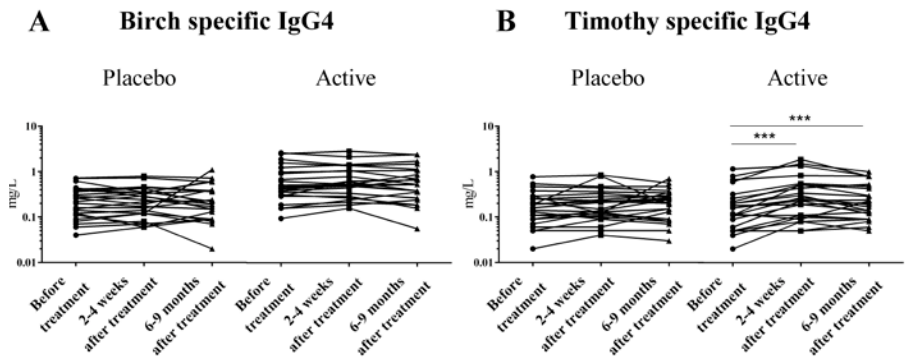


Figure 12. A. The birch specific IgG4 levels did not change in the active or in the placebo group. **B.** The timothy specific IgG4 antibodies increased after active ILIT (n=24) but not after placebo (n=25). ***p<0.001.

4.3 Paper III- High dose grass pollen intralymphatic immunotherapy: two RDBPC trials question the benefit of dose increases

In these studies, ILIT with up-dosing protocols was evaluated in two RDBPC trials; "ILIT after SCIT- 10 000" and "ILIT de novo-3000".

In ILIT after SCIT- 10 000, 29 patients that had recently ended SCIT treatment for grass induced AR were included. The patients were randomized double-blind to three intralymphatic injections of placebo or grass allergen extract in increasing doses; 1000- 3000- 10 000 SQ-U, with injections every 4-6 weeks. All the patients that started the treatment completed the protocol, without any moderate or severe adverse events, and could be included in the analysis.

The primary outcome measure was the daily CSMS at the pollen season, expressed as AUC for the entire study period. The CSMS was reduced by 31% in the active group at the pollen season after treatment compared to the year before treatment. The CSMS in the placebo group did not change (Fig. 13). The between groups analysis of CSMS in the active versus the placebo group did not reveal any difference, although a comparison of the change in CSMS from the baseline season to the post treatment season, in the active versus placebo group, showed a trend for more improved scores in the active group (p=0.059, unpaired t-test). Among the secondary outcomes, the MS improved the year after treatment in the active group but not in the placebo group. Grass-specific IgG4 antibodies were increased in the active group after treatment, whereas the placebo group displayed a steady decline in specific IgG4 after the withdrawal of SCIT (Fig. 14).

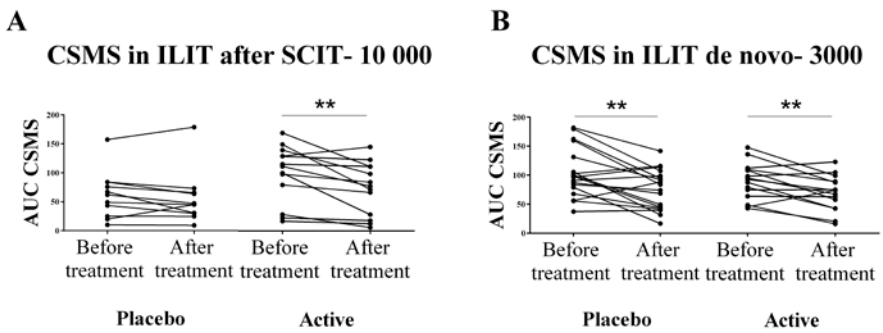


Figure 13. A. In ILIT after SCIT, the CSMS was improved in the active group (n=13) after treatment. The placebo group (n=11) did not show any change. **B.** In ILIT de novo- 3000, the placebo (n=18) and the active group (n=15) scored an equal improvement in CSMS after treatment. **p<0.01.

In ILIT de novo- 3000, 39 patients with moderate to severe AR due to grass pollen allergy were recruited. These patients had not undergone AIT previously. The same dose-escalation protocol as in ILIT after SCIT- 10 000 was attempted but could not be followed. The first two patients that received the 5000 SQ-U dose had anaphylactic reactions. The adverse events were treated successfully and the patients could be discharged from the hospital the same day. The up-dosing was interrupted for the remaining patients. They instead received a third 3000 SQ-U dose resulting in the modified protocol of 1000-3000-3000 SQ-U.

19 placebo treated patients could be compared to 16 actively treated patients in the efficacy analysis. At the time for the evaluation, one year after the vaccination, the pollen levels were markedly lower than during the baseline year. This was reflected as improved CSMS, SS and MS scores in both the active and the placebo group (Fig. 13). However, at the peak pollen season, the placebo group but not the active group showed improved scores. Similarly, the quality of life at the peak pollen season was improved in the placebo group but not the active group. Grass-specific IgG4 levels, but also grass specific IgE levels, were increased 4 weeks after treatment (Fig. 14). Flow cytometry analysis on lymph node aspirations revealed increased expression of activation markers on DCs (Fig. 15). Increased expression of CD86 was positively correlated to a favorable treatment outcome expressed on VAS. The proportion of memory cells in the lymph nodes was unchanged and the amount of Th1 cells and T reg cells in blood was not increased.

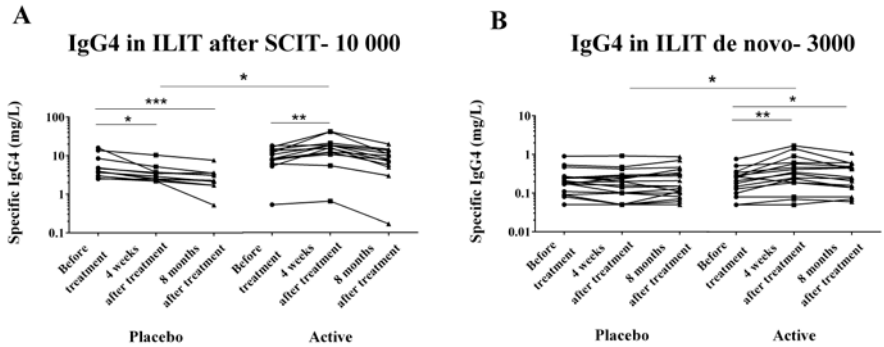


Figure 14. A. Grass specific IgG4. In ILIT after SCIT the grass specific IgG4 levels decreased in the placebo group during the course of the study (n=11), while the active group had a boost in the specific IgG4 levels after treatment (n=13). **B**. In ILIT de novo- 3000, the active group showed a moderate increase in grass specific IgG4- levels (n=16). The placebo group did not change (n=19). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

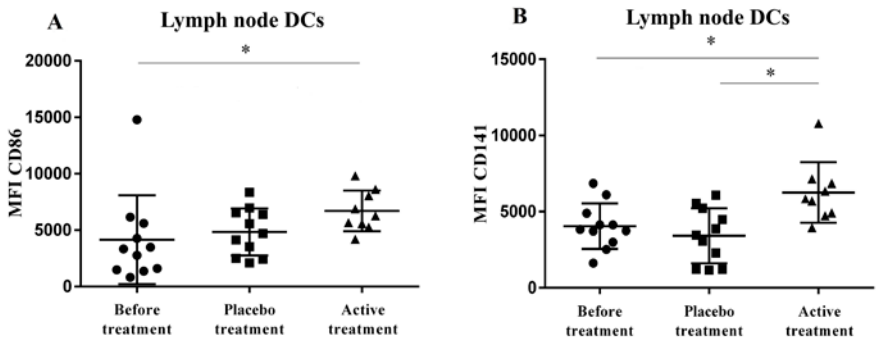


Figure 15. Activation of DCs from the lymph nodes in ILIT de novo-3000. DCs displayed an increased expression of **A.** CD86 and **B.** CD141 in the active group (n=9) but not in the placebo group (n=11). *p<0.05. The outcome after treatment was compared to pooled data from the active and the placebo patients (n=11) before treatment.

4.4 Paper IV- A five-year open follow up of a randomized double-blind placebo-controlled trial of intralymphatic immunotherapy for birch and grass, reveals remaining beneficial effects

20 out of 25 eligible patients from the active arm in the previous RDBPC-trial (paragraph 4.2 above) could be enrolled in an open long-term follow-up study. In the placebo group, only 8 out of 28 patients could be included; 8 patients had undergone AIT, 5 patients had moved away and the remaining did not give consent. 6 new patients with birch and grass pollen induced AR were included in the study and analyzed together with the placebo patients, as a non AIT-treated control group.

The primary outcome measure was reactivity following grass allergen NPT. The analysis could not detect any remaining effect on NPT in the active group, compared to baseline before treatment (Fig. 16A). When comparing grass NPT in the previous active ILIT group with the non-AIT treated control group, there were lower scores in the ILIT-treated group (Fig. 16B). The birch NPT did not reveal any differences between the groups. The modified seasonal CSMSs were lower in the active ILIT-group compared to the non AIT-treated group, at the birch and the grass pollen seasons (Fig. 16). When examining the medication use separately, there were lower MSs at the birch and grass pollen seasons in the active group. The SSs did not differ between the groups.

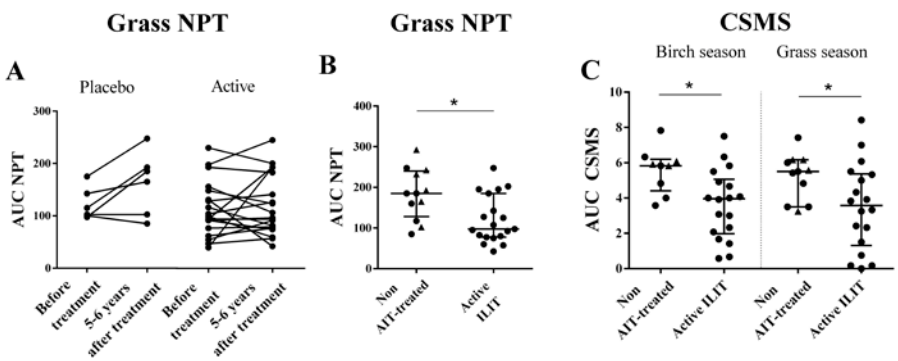


Figure 16. A. The reactivity at grass NPT was the same 5-6 years after ILIT compared to baseline in the placebo (n=6) and the active (n=19) group. **B**. The NPT scores 5-6 years after treatment were lower in the ILIT-treated group (n=19) compared to the non AIT-treated control group (n=12). C. The CSMSs were lower in the ILIT-treated group (n=18) compared to in the non AIT-treated group (n=9-11). *p<0.05. **B-C.** Horizontal lines at median and interquartile ranges. Dots represent patients from the previous RDBPC-study. Triangles represent new AR-patients without previous AIT or participation in the RDBPC-study.

The active group, that had an increase in grass specific IgE directly after ILIT-treatment, had 5-6 years later lower specific IgE levels compared to baseline. At the time of the follow up a detectable increase in the levels of grass specific IgG4 antibodies could still be seen (Fig. 17). Birch specific IgE and IgG4 antibodies were unchanged compared to levels seen before treatment. Flow cytometry revealed higher amounts of B-cells in the lymph nodes of previously ILIT-treated patients compared to the control group. The total amount of T-cells in the lymph nodes were the same but the ILIT treated group showed a larger proportion of memory cells than the non-AIT-treated group.The previously seen differences in the levels of Th1 cells and Treg cells in blood in the ILIT treated group could not be reproduced.

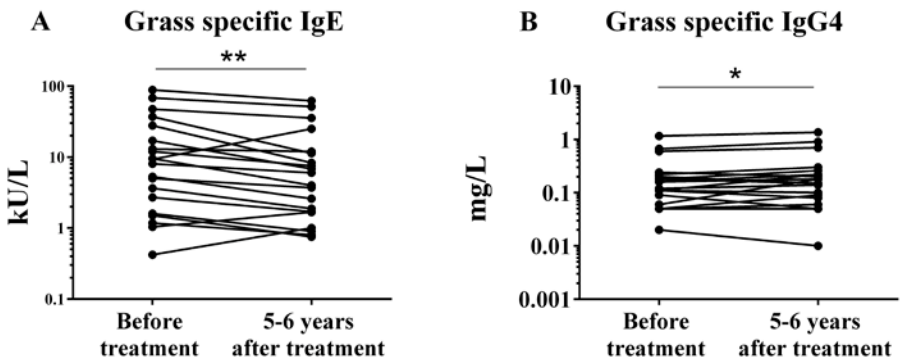


Figure 17. A. The grass specific IgE levels had decreased 5-6 years after ILIT (n=20). B. The grass specific IgG4 levels were slightly increased 5-6 years after ILIT (n=20). *p<0.05, **p<0.01.

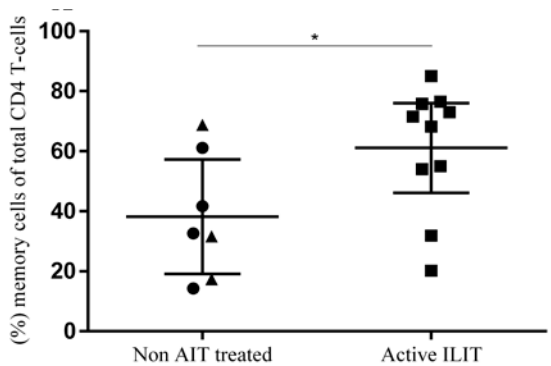


Figure 18. Patients treated with active ILIT displayed an increased fraction of CD4 memory T-cells. *P<0.05, Horizontal lines represent the mean value and SD. Triangles represent new AR-patients without previous AIT or participation in the RDBPC-study.

5 DISCUSSION

5.1 Safety

One of the main advantages of ILIT is the possibility to use lower allergen doses than in other forms of AIT. The allergen is administered directly into the lymph nodes. They contain a high abundance of T-cells and B-cells, which hypothetically confers a high chance of allergen specific immune reactions even with low concentrations of the allergen. In addition, the lymph nodes contain relatively few mast cells and basophils, reducing the risk of immediate side effects further.

5.1.1 Injection technique

The goal is to deliver all the allergen into the lymph node guided by ultrasound, but not all injections are successful. Occasionally, some of the allergen is deposited just outside the node. This can be related to leakage from the node or inadequate placement of the needle. The former could, to some extent, be prevented by a slow injection. Sometimes the lymph node is surrounded by a rather tough capsule that can be difficult to penetrate and the allergen is deposited in the immediate vicinity. Injection of allergen outside of the lymph node most likely increases the risk for local adverse reactions and may, in the extreme situation, if erroneously placed into the femoral vein or artery, be life threatening. In doubt of the needle position it is advisable to aspirate before the injection to rule out intravascular position. Pre-requisites for an optimal ILIT injection include a comfortable patient that can refrain from moving during the procedure, good ultrasound visualization, a slow injection with or without preceding aspiration.

5.1.2 Allergic adverse events

5.1.2.1 Dosing schedules up to 1000 SQ-U

We used the same doses as in previous ILIT studies, three injections of 1000 SQ-U ALK Alutard® with one-month intervals (paper I-II) (52-54, 59, 61). No serious adverse reactions were experienced. Our studies confirm the notion that this ILIT schedule is safe, also when used for two allergens, birch and grass, given concomitantly, with 30 minutes in between. It is important to notice that uncommon reactions might occur and that it can take several years of use before they can be revealed. There will probably never be an AIT treatment involving crude allergens, as Alutard®, without a risk for side effects. Hence, the same rigorous routines for monitoring patients after the injections as in SCIT should therefore always be practiced also in ILIT.

In paper I, the participants had to have a confirmed history of mild asthma in addition to AR. There were no acute obstructive reactions in conjunction with the injections, but one patient reported asthma symptoms during the day after one of the injections. All patients were well controlled with a median FEV1 of 97.5%, a median F_{ENO} of 20.5 and median ACT of 21 at enrollment. It is possible that the safety profile is not as good among patients with more severe asthma, as in the present study. In paper III, ILIT de novo- 3000, perennial asthma constituted an exclusion criterion, whereas seasonal asthma was permitted. Two patients reported heavy breathing as a late reaction after the first 3000 SQ-U dose. Both patients had seasonal asthma but denied persistent symptoms and had a normal lung function test with FEV1 >80%. One of the patients was withdrawn from the study after that reaction. The other patient continued the up-dosing to 5000 SQ-U at which he experienced an anaphylactic reaction. Hence, special caution should be taken with asthma in ILIT (11).

In paper II, polysensitized patients were treated with two allergens, birch and grass. No serious systemic adverse events were recorded. Among 30 active patients there were 12 local reactions at the injection site (40%). This is low compared to local side effects reported in SCIT. According to a systematic review in 2007, pooling results from 30 RDBPC trials, 834 events of local reactions occurred among 907 active patients (92%) (109). In a more recent review from 2017, 26-86% of all patients that received SCIT experienced large (> 2.5 cm) local reactions (110). Hence, ILIT with injections of one or two allergens in the dose 1000 SQ-U at one-month-intervals seems to induce fewer local and systemic adverse reactions than regular SCIT.

5.1.2.2 Dosing schedules up to 10 000 SQ-U

The tolerance inducing effect of AIT is, according to the prevailing paradigm, dependent on a high dose of allergen to mount a tolerogenic immune response (111). Patterson et al. have performed "high" up-dosing of ILIT in seven patients without adverse reactions (112). That study used Center-AL®, which is an aluminum adsorbed allergen extract available in the USA. The up-dosing reached 250 PNU (protein nitrogen units), which corresponds to the 5th dose of an 11 weeks up-dosing schedule for SCIT with Center-AL®. We used Alutard® to gain a corresponding dose increase in our ILIT studies. The protocol chosen of 1000-3000-10 000 SQ-U was estimated to be in the same range as the Center-AL® to 250 PNU, even though a direct comparison of these extracts is hard to make.

Dose escalation to 10 000 SQ-U was safe, probably due to the tolerance induced after the recent SCIT treatment (Paper III). These patients had completed three years of SCIT including both up-dosing and maintenance. Not more than 20 months was allowed between the last maintenance dose of SCIT and the first ILIT dose.

An extended time frame could perhaps have been accepted, since it is widely acknowledged that the reduction of airway symptoms upon SCIT is much longer than three years and symptom reduction is believed to reflect tolerance induction (13). Accordingly, a too long period between SCIT and ILIT might be risky, since the protective effect after SCIT declines with time.

An up-dosing of grass allergen from 1000 to 3000 SQ-U among previously unvaccinated patients appeared to be safe (ILIT novo- 3000). In contrast, further dose escalation to 5000 SQ-U, triggered anaphylactic reactions in two out of two patients. Since the state of readiness was high these complications could be handled without delay and the patients released from the hospital within 4-6 hours. Nevertheless, we found these reactions to be a bit surprising since the dose escalation from 3000 to 5000 SQ-U corresponds to half a logarithmic step, a step size commonly used during the traditional up-dosing in SCIT.

Various explanations for these anaphylactic reactions could be contemplated. First, the extract used at the 5000 SQ-U doses, was prepared by diluting the allergen to the final concentration 50 000 SQ-U/mL. An error in the mixture might have happened, but the procedure was carried out with meticulous measurements and an erroneous allergen content in the final vial is not likely. Further, the two patients that encountered the anaphylactic reactions might have been hypersensitive to the allergen used. One of the patients had reported heavy breathing and palpitations 4-8 hours after the injection already at 3000 SQ-U, which was noted but assessed as unspecific. This might represent a late systemic reaction signaling a risk for hypersensitivity reactions in this particular patient, if interpreted with caution. At the same time, the other patient did not experience any side effects after the preceding injection and yet had an anaphylactic reaction at injection number three. A third possibility is that the allergen after the injection was drained fast to the hilus of the lymph node and further to the venous system via the thoracic duct, causing the rapid systemic reaction. If so, this might be prevented by an even slower injection.

It is interesting to notice that the two patients that received 5000 SQ-U did not display any signs of symptom improvement during the pollen season, nor did the other patients in the active group that were up-dosed to 3000 SQ-U. It is therefore tempting to conclude that higher doses than 1000 SQ-U in ILIT, with dose intervals of 1 month, fail to induce tolerance, resulting in a lack of clinical effect and a severe risk for anaphylaxis.

5.1.3 Non-allergic adverse reactions

5.1.3.1 Infections

One placebo patient in study II had clinical signs of epididymitis 1 week after the last injections. Hypothetically, a retrograde infection from the inguinal lymph node could be the cause of this but seems unlikely considering the drainage of lymph fluid. One placebo patient in study III developed fever and swelling in the groin after the second injection. This resolved spontaneously within 24 hours, before it was reported to the study staff. It might represent a local injection-related infection, but the etiology is hard to determine in retrospect. Infections due to the injections are extremely rare in SCIT (21). Injections in the inguinal region are possibly more prone to be contaminated. Aseptic technique should be used.

5.1.3.2 Autoimmune diseases

One patient in study I was diagnosed with sarcoidosis after the booster injection. Investigations including analysis of blood samples and bronchoalveolar lavage could neither confirm nor rule out a connection to ILIT. Sarcoidosis is an inflammatory disease of unknown etiology, with overlapping pathogenesis with autoimmune disorders, including clusters of activated T-cells. Sarcoidosis-like manifestations have been described in conjunction to other immune modulating drugs (113). Sarcoidosis is a relative contraindication for SCIT since the Alum adjuvant have been suspected to cause nodular granuloma formation at the injection site in sarcoidosis patients (114). To the present knowledge, AIT is not a risk factor for sarcoidosis or other autoimmune disease (114), although one case report has suggested that the responses following AIT might be one of several predisposing triggers (115). The fact that the present patient did not suffer from lymph node engagement in the inguinal region, might speak against a causal relationship.

5.2 Clinical effect

5.2.1 Three ILIT doses of 1000 SQ-U (paper I and II)

5.2.1.1 Medication use

The outcome of three 1000 SQ-U doses of ILIT indicated improvement of AR demonstrated as less need for symptom relieving medication during the subsequent pollen season. The patients evaluated their use of medication after each pollen season using a modified scoring system (paper I), and by categorizing the need for symptom relieving medication as reduced, unchanged or increased (paper II). Both studies found a reduction of these scores. This finding is in line with the first ILIT-study from Senti et al. that used grass allergen in the same doses (52). They reported that the number of antihistamine tablets used during the grass pollen season was lower after ILIT.

The scores in study I represent pooled results from birch and grass ILIT, which is a limitation. However, most of the patients received birch ILIT which implies that also birch allergen ILIT is effective. Study II included an open booster injection at the consecutive year in the active group which reduced the medication use further during year two, but these findings are preliminary and in the absence of a control group.

5.2.1.2 NPT

The primary outcome in both studies was the reactivity as measured by NPT. In study II, a reduction of the rhinoconjunctivitis symptoms was seen in the active group. A 28% improvement In NPT reactivity cannot be directly translated to a corresponding level of protection at seasonal exposure, but it incontrovertibly argues for a positive effect of ILIT. NPT with birch allergen was not performed due to logistical reasons and this is a limitation in the study. One drawback of NPT is the well described high variability of the test (86), which confers the need for a large sample size to detect changes. This might explain why it was not possible to detect any reduction in NPT symptoms in study I, that had a smaller sample size.

Improvement of NPT scores have also been demonstrated in other ILIT trials. The first ILIT trial of Senti et al. (52), that also used grass pollen allergen, demonstrated increased thresholds at NPT, as did a subsequent smaller open ILIT trial of grass allergen (53). Other ILIT trials include a recently published RDBPC study of Japanese cedar pollinosis. The active cedar group showed less symptoms during three years of repeated NPT:s at follow up visits, in comparison to baseline. The placebo group did not improve. However, the placebo group was smaller than the active group since a 1:2 allocation ratio was used. This undermines the possibility to compare the changes within each group. Another RDBPC trial that used a modified cat epitope allergen (58) revealed increased tolerance to nasal allergen after active ILIT compared to placebo.

In summary, several ILIT trials have shown reduced NPT reactivity with various allergens. The study in paper II is the first RDBPC trial that demonstrates improvement at grass allergen challenge after ILIT.

5.2.1.3 Seasonal symptom improvement

In study I, we studied if ILIT also affects asthma favorably. The asthma parameters turned out to be unchanged after treatment. This is most likely explained by the fact that all patients were on optimal asthma treatment at enrollment with well controlled disease. No attempt to withdraw medications was performed so there was little room for improvement. In study II, the active patients reported a reduction in the use of β2-agonist inhalations during the birch pollen season after treatment, a finding that might suggest a protective effect on bronchial obstruction.

None of the studies with ILIT 1000 SQ-U could verify an improvement on seasonal AR symptoms that exceeded the treatment effect in the placebo group. In study I, the recalled SS was reduced in both the active and the placebo group. In study II, the active and the placebo group estimated an equal treatment effect on VAS. In other respects, the intra-seasonal scoring of AR related QoL in study II showed a trend towards a clinically relevant improvement during the birch pollen season. In fact, there was a significant improvement in the active group compared to the placebo group in some domains, e g nasal symptoms.

Generally, the evaluation of symptoms scored after the pollen season is hampered by the risk of memory bias and can therefore only be a rough estimation of the outcome. We have previously, in a smaller ILIT study from 2013 (59) found an improvement on VAS. This disparity might in part be related to differences between the recruited populations. The 2013 study enrolled patients from the waiting list for SCIT with severe AR. The participants in paper I and II were recruited from the pediatric asthma clinic and from newspaper advertising, possibly with a lower grade of disease severity. Evaluation of the AIT outcome in highly symptomatic patients is more likely to demonstrate a positive effect, than among patients with less pronounced symptoms (116).

5.2.1.4 Lessons learned of use for our subsequent ILIT studies

The reduction of NPT induced rhinoconjunctivitis symptoms (paper II) and the diminished consumption of symptom relieving medication (paper I and II), provided evidence for a good ILIT efficacy with three doses of 1000 SQ-U. The RDBPC study design is a strength in our studies and the relatively small sample sizes and blunt evaluation tools are weaknesses. The latter might be one explanation to why we could not convincingly prove an effect on the seasonal symptoms. As stated before, the effect of AIT is generally believed to increase if higher doses of allergen can be given. Therefore, the next ILIT trials described in paper III were designed as an up-dosing study. We also sharpened the inclusion criteria regarding disease severity and switched the primary outcome measure to the CSMS.

5.2.2 ILIT in doses higher than 1000 SQ-U (paper III)

The two trials in paper III, “ILIT after SCIT- 10 000” and “ILIT de novo- 3000” investigated a dose escalation protocol that aimed at enhancing the clinical effect of ILIT.

5.2.2.1 ILIT after SCIT- 10 000

In ILIT after SCIT- 10 000, patients with grass pollen induced AR that had recently completed a SCIT treatment without full symptom control, received ILIT in doses 1000- 3000- 10 000 SQ-U. The year after treatment, the CSMS was reduced by 31%, the MS was reduced by 52% but the symptom score SS was unchanged.

The World Allergy Organization recommends that the primary outcome measure in AIT trials should compare the CSMS in the active versus the placebo group and the relative difference should exceed a 20% improvement(117). Neither the between group comparisons of CSMS in the active vs the placebo group, nor the change in CSMS, reached statistical significance in ILIT after SCIT- 10 000 and this is a weakness. Comparing two seasons with different pollen burden may introduce a bias due to the variability in allergen exposure. In the present study, the pollen counts were indeed 17-21% lower during the posttreatment year compared to baseline. Despite this, the placebo group did not improve the CSMS, which might illustrate a gradual decline of the symptom control gained at previous SCIT and advocates a true improvement in the active group.

Two previous studies have monitored the efficacy of AIT for grass pollen induced AR by comparing two consecutive grass pollen seasons (118, 119). The first study presented improvement on VAS as well as intra-seasonal symptoms and medication use after SLIT. The second study demonstrated a significant difference in the change of AUC of CSMS comparing 132 active patients versus 49 placebo patients, in favor of active grass SLIT. The secondary outcomes SS and MS were also improved. These findings derived from a 39% absolute reduction of CSMS in the active group to 37% reduction of SS and 41 % reduction of MS.

The calculation of medication weighted symptoms have varied between the previously formed AIT- trials (120) which make direct comparisons of the improvement difficult. Systematic reviews estimate that the treatment effect in different CSMS, relative to placebo, in recent and well powered SCIT and SLIT trials range from 26 to 36 % (24). A systematic review of the treatment effect of grass SLIT treatments determined the reduction in MS, relative to placebo, to 27-38% (121). Moreover, when comparing SLIT to symptom relieving pharmacotherapy treatment alone, the improvement in total nasal symptom scores (comparable to SS) relative to placebo was 16% for SLIT, 9% for Desloratadine (antihistamine tablet) and 22% for intranasal steroid spray. Lastly, a recent SLIT trial using the exact same CSMS scoring system as we did in ILIT after SCIT-10 000, exhibited a 32% reduction of CSMS in relation to placebo (122).

Secondary outcome measures did not support the findings in ILIT after SCIT-10 000, drawbacks of each method have been discussed earlier. To note, the patients in ILIT after SCIT- 10 000 had lower NPT scores at baseline compared to untreated patients. The allergen dose used at the provocation might be too low among SCIT-treated patients to elicit a reaction that can be improved after ILIT.

When comparing these previous AIT-trials to ILIT after SCIT- 10 000, the result with 31% improvement of CSMS within the active group is probably somewhat below the effect of established AIT-forms but might exceed the minimal clinically

important difference of 20% in relation to placebo. The absolute MS reduction of 51% seems to be in the same range as other AIT-treatments that have 27-38% reductions relative to placebo, but since the patients in our study had been SCIT-treated the different study populations might play a role, as well as limitations in the statistical methods with a small study sample. Nevertheless, aiming at finding new cost-effective treatment forms for AR, these results are encouraging.

5.2.2.2 ILIT de novo- 3000

The key question of the twin studies in paper III was to investigate whether an up-dosing of ILIT could further improve the therapeutic effect in previously unvaccinated patients, at least in doses up to 3000 SQ-U. Disappointingly, the results did not support any improvement. The active group did not exhibit improvements in any of the parameters including the daily scoring, RQLQ, VAS and NPT. If anything, the non-treated group seemed to enjoy a placebo effect with improved CSMS and RQLQ during the heights of the pollen season, which the actively treated patients did not. This might even suggest an aggravation of the allergic symptoms among actively treated patients. Even though the sample size of this study was limited, a larger treatment effect could not have remained undetected.

5.2.3 Long term effect of ILIT in 1000 SQ-U (paper IV)

Study IV was an open follow up study 5-6 years after the RDBPC ILIT trial for birch and grass induced AR (paper II). The follow-up could not verify any long-term effect on sensitivity at NPT, which was the pre-specified primary outcome measure. However, the comparisons of CSMS and NPT revealed that the scores were lower in the ILIT treated group than in the control group consisting of previous placebo-ILIT treated patients and newly recruited AR-patients. This suggests a long-term protective effect of ILIT.

Limitations of this study include the open study design. However, breaking the study codes after the first season in the RDBPC trial was the only option in order to recruit participants. Secondly, all patients in the control group had not been previous participants in the RDBPC trial. Thus, they were not allocated randomly to the control group. Differences between the previously ILIT treated group and non-AIT treated control group might therefore be attributed to other factors than the treatment. The new patients in the control group might have had a more severe AR compared to baseline values of the active group. Nevertheless, examining the CSMS and NPT scores visually revealed that the control patients are not outliers in comparison to the previously placebo treated ILIT patients (Fig. 16 B-C).

At the follow up visits, many patients stated that the effect of ILIT had lasted for 3 years. This is in line with the first ILIT trial 2008 and a recent RDBPC trial

of Japanese cedar pollinosis, that measured improvements during 2-3 years (52, 123). SCIT and SLIT usually require 3 years of treatment to display long-term effects at least two years after completed treatment. Two years of treatment is often insufficient to reach a sustained effect after the treatment withdrawal and 1 year of treatment is sometimes not enough to achieve short term improvement at the first season (12). In the light of this, the now often standardized ILIT protocol of three visits during 8 weeks might be supplemented by yearly pre-seasonal booster injections during e g three years, harmonizing the total treatment period of ILIT with other forms of AIT.

5.2.4 Summary of treatment effect

In summary, our presented data strongly support a good clinical effect of ILIT given as 1000-SQ-U during 8 weeks. Further, we found no evidence for that a further increase of the dose should be beneficial. Rather the contrary, higher doses might be decremental with loss of clinical effect and increased risk for severe side effects. In the special case when a higher dose, 10 000 SQ-U, is given to an already SCIT treated population therapeutic gains might be reached without compromising the safety. When considering ILIT as a future alternative in AIT it is important to acknowledge that many of the early SLIT studies failed to verify a positive effect, hampered by unknown factors such as optimal dose, type of preparation, duration of treatment and mode of intake (124). It seems as ILIT presently might be in the same developmental position as SLIT was 10-20 years ago.

5.3 Mechanisms in ILIT

5.3.1 Allergen specific IgE and IgG4

We observed increases in the allergen specific IgE and IgG4 levels shortly after administration of active treatment (paper I-III). The follow up 5-6 years after 1000-SQ-U ILIT, presented in paper IV, revealed that the timothy specific IgE had decreased to levels below the baseline values. Similarly, the levels of timothy specific IgG4 had returned towards baseline but were still slightly elevated compared to before treatment. This suggests a favorable induction of IgG4 producing B-cells with a short onset and long-term duration. Other ILIT studies have demonstrated increased IgG4 levels specific for cat dander, house dust mite and grass (56-58, 61) even though the grass-ILIT trial of Witten et al. (61) did not prove a clinical improvement. These serologic findings are in line with findings in conventional AIT, with an increase in allergen specific “blocking” IgG4 antibodies upon treatment. The transient increase in the timothy specific IgE levels, followed by a gradual decline, also mimics the pattern induced by other forms of AIT (20, 125, 126). One ILIT-study could not detect any boost in allergen specific IgG4

but found an increase in plasma cells producing non-IgE antibodies, suggestive of a shift in antibody production (53).

The changes in timothy specific IgE and IgG4 responses in paper II and IV were not mirrored by changes in antibodies specific for birch. This might be an effect of differences in the relative allergen content between the two allergen extracts used (127). This discrepancy might be neglectable in SCIT but have more impact in ILIT where the total doses used are far lower. However, the pooled birch and grass allergen specific IgG4 results in paper I did show an increase, supporting evidence of a birch specific IgG4 class switch.

The timothy specific IgG4 levels in ILIT after SCIT-10 000 showed a larger increase, starting at a higher baseline level, than in the 1000 SQ-U studies. This difference in baseline levels can be attributed to an expanded memory B-cell population with inducible IgG4 production after the previous SCIT. ILIT de novo- 3000 boosted the timothy specific IgG4 levels, to the same extent as in paper II, but without accompanying symptom relief. So, it is tempting to conclude that complex mechanisms involved in successful AIT involves other or additional cellular changes, that might not have been achieved when using higher doses of ILIT.

5.3.2 T-cells

In lymph nodes we found an induction of effector memory T-cells and an increase of Treg cells and Th1 cells in blood (paper II). These findings were not seen at the follow-up 5-6 years after treatment (paper IV). Instead, the cells from the lymph nodes contained a higher proportion of total memory cells than the untreated group, with a tendency of more central memory T-cells in the active group compared to the placebo group.

The expansion of IgG4 instead of IgE producing B-cells upon AIT is believed to be orchestrated mainly by allergen specific regulatory Tregs secreting IL-10 (20). It is possible that the increase in lymph node derived effector memory cells is partly constituted of newly induced allergen specific Treg cells on the way to migrate to the periphery. One can also speculate that the increased level of central memory T-cells seen are in part allergen specific long-lived memory cells residing in the lymph nodes prepared to proliferate at a recall signal e g during the pollen season. However, the low amount of lymph node material available after aspiration limited the analyses that could be performed.

Previous studies have investigated changes in T-cells in blood after ILIT. In a study of modified cat-dander allergen increased activation and proliferation of T-cells was seen 9 weeks after the start of the treatment (1 week after the last injection). The T-cells then turned unresponsive for allergen stimulation 1 year after treatment.

Moreover, reduced amounts of allergen specific T-cells, increased levels of IL-10 and FOXP3 were demonstrated (79). This study is special since it involved a transporter molecule to enhance intracellular signaling and therefore the same immunological changes can perhaps not be expected in other trials. Nevertheless, other favorable T-cell changes were reported in a pilot study of grass or birch- ILIT, where the levels of Th2 cells decreased and Treg cells and IL-10 increased during the treatment (54). A cedar tree ILIT trial searched for Treg induction after the third pollen season but could not identify any long-term changes (123). In a short dose-interval ILIT-study without signs of clinical improvement, reduced levels of IFN-γ was noted, suggesting a reduction of Th1 activation (61).

5.3.3 Basophils

In an attempt to characterize a third domain of tolerogenic response after ILIT, analyses of basophil characteristics were performed (paper IV). The basophils in the active ILIT group exhibited a low density of the high affinity receptor FcεR1 on the surface, and a corresponding low density of membrane bound IgE. Expression of FcεR1 on basophils is known to be induced at higher IgE levels in serum (105). The timothy specific IgE levels were higher in the non-AIT treated group, consequently, our findings could be a result of this correlation. On the other hand, a recent study that characterized the FcεR1 on basophils during SLIT, did not find any difference in the expression of FcεR1 in AR-patients with low disease severity and low specific IgE levels, compared to a subgroup with more pronounced symptoms (105). Moreover, the transient increase in specific IgE upon SLIT did not correspond with an increase in FcεR1 expression during the first year of treatment. Reasons for the discrepancies between our results and the findings in the mentioned study, might be attributed to the long-term treatment effect after ILIT, disparities in IgE levels and differences in the timing of the sampling.

We also performed a simplified basophil reactivity test (paper IV). This revealed a trend towards a lower degree of CD63 expression upon timothy stimulation in the active ILIT group than the non AIT-treated group. However, the relevance of this finding is uncertain since, in addition to not being significant, it was only measured after stimulation with one allergen concentration and not as a series of dilutions (103). The concentration chosen (500 SQ-U) is relatively high, leading to a high degree of activation among most sensitized patients. Stimulation with lower allergen concentrations might have demonstrated more prominent differences, based on the typical dose-response curve seen in BAT(128). We did not have any baseline values of the basophil reactivity and could not determine longitudinal changes, which is another limitation.

Basophil reactivity testing can be used in allergy diagnostic and reduced reactivity is often seen early in SCIT, predicting symptom relieving effects (20, 103). In a previous ILIT study of grass, the basophil activation was not changed during the treatment or directly after the pollen season (53). This might be explained by factors associated with the exposure to allergen during treatment and soon after the pollen season. It might also indicate that the mechanism in ILIT differs, at least partly, from SCIT and does not involve basophil changes (53).

5.3.4 DCs

DCs residing in the tonsils are believed to play a pivotal role in tolerance development during SLIT (20). This is supported by findings of clusters of DCs and Tregs in the tonsils and the capacity of DCs to induce allergen specific Treg cells (129). We investigated the role of DCs in the ILIT de novo-3000 study. Discouragingly, the modest dose increase from 1000 to 3000 SQ-U did not show any clinical benefits. The DCs in the lymph nodes did exhibit increased level of activation characterized as expression of CD80, CD86 and CD141. However, the latter is attributed to Th2 activating properties (130) which is in line with the lack of a clinical improvement.

5.3.5 Lack of clinical effect of 3000 SQ-U

There were no signs of clinical improvement in ILIT de novo- 3000. This was unexpected since the use of high allergen doses in conventional AIT generally is believed to produce a better symptom relief (111). This outcome also stands in contrast to the improvement induced by up-dosing in ILIT after SCIT- 10 000. It seems that this does not apply to unvaccinated patients. In some way the allergen dose might be too high to be favorably processed inside the lymph node when not actively transported to the site by antigen presenting cells. The aluminum hydroxide adjuvant in Alutard® when given in larger amounts might be too immunogenic. Alum has been shown to have Th2- skewing properties (131), potentially counteracting tolerance induction. The role of alum in ILIT has not been described, it might be either unnecessary in ILIT, or crucial for the immunogenicity. Further, allergen specific B-cells residing in the lymph nodes might take up allergen after injection. When presenting very high amounts of antigen, the B-cells might be excluded from entering the follicular zones to proliferate and instead become anergic, in analogy with the peripheral selection process concerning elimination of self-antigens (132).

Taken together, it is tempting to conclude that both an optimal dose and a well selected time interval is necessary for a positive outcome of ILIT. A too high dose or a too short time interval might be decremental. It is also important to notice that time and dose is closely related. A shorter time interval results in a higher

effective dose per week. Hence, our lack of symptom relief with the a higher ILIT dose can be related to the outcome in the study of Witten M, et al. 2013. They failed to demonstrate clinical effects despite allergen specific IgG4 induction (61). This trial used the same doses as most other ILIT-studies, 1000 SQ-U, but shortened the dose intervals to 2 weeks, leading to a higher allergen dose per time unit. Hypothetically, a dose increase in ILIT should be accompanied with a prolongation of the dose interval beyond 4 weeks. This could allow periods of low allergen content in the lymph node which has been speculated to be important for tolerance development in ILIT (62). This does not provide any explanation to the positive clinical improvement in ILIT after SCIT- 10 000. In some way, the previous SCIT treatment seems to have primed the immune system to be ready for tolerogenic responses at ILIT.

6 CONCLUSIONS

- ILIT with birch or grass allergen given in three doses of 1000 SQ-U with one month apart seems safe among young patients with well-controlled asthma. Two allergens, birch and grass, can be given concomitantly using the same schedule. Higher doses of ILIT (1000- 3000- 10 000 SQ-U grass) can be given as add-on in previously SCIT vaccinated patients. However, the same schedule causes severe systemic problems in unvaccinated patients, hence doses exceeding 3000 SQ-U should not be used.

- An improvement of allergic asthma symptoms as a result of ILIT among patients with already well controlled disease was not possible to detect.

- Three ILIT-injections of 1000 SQ-U with birch and grass allergen given concomitantly reduce the symptoms at nasal grass allergen provocation and the need for seasonal symptom ameliorating medication.

- ILIT with grass allergen in escalating doses of 1000- 3000- 10 000 SQ-U after SCIT reduces the combined symptoms and medication scores. A schedule of 1000- 3000- 3000 SQ-U in previously unvaccinated patients failed to induce any form of clinical improvement.

- A 5-6-years follow up of patients treated with grass and birch concomitantly (1000- 1000- 1000 SQ-U) revealed remaining low seasonal combined symptoms and medications scores, reduced grass specific IgE levels and produced a small sustained increase in grass specific IgG4. Altogether this indicates the possibility of long-term clinical effects.

- A short termed positive clinical outcome was associated with an increase in allergen specific serum IgG4 levels, an amplification in the proportion of memory T-cells in the lymph nodes with an augmented ratio of effector memory/central memory T-cells. In addition, high levels of $CD4^{+}CD25^{++}$ regulatory effector memory T cells and $CD4^{+}CCR5^{+}$ Th1 type of central memory T-cells in blood was noted. A positive long termed outcome seems to be signified by an increased proportion of B-cells and memory $CD4^{+}$ T-cells in the lymph nodes, in combination with reduced grass specific IgE levels in serum.

7 POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Allergisk rinokonjunktivit (AR), eller ”hösnuva”, är en vanlig sjukdom i västvärlden. Närmare 40% av den yngre delen av befolkningen är drabbad. Hos den som drabbats utlöser i grunden harmlösa ämnen, som pollen, pälsdjur och kvalster, en allergisk reaktion relaterad till förekomst av IgE-antikroppar. Rinnande och kliande ögon samt snuva, nysningar och nästäppa utgör sjukdomens huvudsymptom. Inte sällan förekommer också symptom på periodisk allergisk astma. Många med AR lider dessutom av påtaglig trötthet, något som varit känt sedan länge men vars betydelse ofta har negligerats. AR medför försämrad livskvalitet med en negativ påverkan på arbete, studier, sömn och socialt liv. Vår grupp har i tidigare studier beräknat att AR kostar det svenska samhället drygt 13 miljarder kronor årligen.

AR behandlas vanligen med antihistamintabletter och kortisonnässpray. Flera undersökning visar dock att en stor del av patienterna inte är helt nöjda med effekten av denna behandling. Speciellt gäller detta livskvalitetssänkande symptom som trötthet. En mindre del av AR patienterna blir föremål för allergenspecifik immunterapi (AIT), s k allergivaccination. Detta är den enda behandling som förutom att den har effekt på symptomen, inklusive trötthet, också påverkar sjukdomens långsiktiga utveckling i positiv riktning.

Vid AIT ges successivt ökande doser av det ämne man är allergisk mot (t ex björk, gräs eller kvalster), som injektion under huden eller som en tablett under tungan. Behandlingseffekten är hos flertalet mycket god och den symptomlindrande effekten varar vanligen flera år efter avslutad kur. Dock är det en tidskrävande behandling med upp emot 50 besök på sjukhuset för sprutor under tre till fem år, eller tabletter dagligen under tre år. Allergiska biverkningar förekommer. Vid tablettbehandling är klåda och svullnad under tungan vanligt och vid injektionsbehandling finns en risk för kraftig allergisk reaktion som astma, nässelutslag och, även om det är mycket ovanligt, allergisk chock. Dessutom är tillgången till allergikliniker begränsad så sammantaget är därför AIT en underutnyttjad terapiform.

Intralymfatisk immunterapi (ILIT) är ett nytt sätt att ge AIT som under senare tid vunnit insteg bland de allergiforskande kliniskt aktiva läkare som strävar att utveckla mer användarvänliga former av AIT. Med vägledning av ultraljud injiceras allergenet rakt in i en av ljumskens lymfkörtlar, där det kan utöva en direkt verkan på immunförsvaret. Med endast tre injektioner under åtta veckors tid har man erhållit en god symtomförbättring som väl motsvarar den man ser vid konventionell AIT. Eftersom det räcker med en mycket låg dos när allergen levereras direkt i lymfkörteln, är risken för biverkningar dock påtagligt lägre än vid konventionell AIT.

Den här avhandlingen syftade till att fortsätta undersöka ILIT genom att kartlägga säkerhet och effekter vid allergisk astma och vid injektion av mer än ett allergen samtidigt, liksom effekterna av en ökad dos av allergen. Vidare studerades om behandling med ILIT ger några kvarstående positiva effekter på allergisymptomen 5–6 år efter avslutad terapi. För att möjliggöra en god utvärdering av effekten lottades patienter till behandling med ILIT eller placebo.

I det första delarbetet såg vi att ILIT vid björk- och gräspollenallergi kan ges från 16 års ålder och att samsjuklighet med välkontrollerad astma inte ökade risken för biverkningar. Användningen av symptomlindrande mediciner under pollensäsongen minskade första året och efter en så kallad boosterdos det andra året minskade medicinförbrukningen ytterligare. IgG4, som är en typ av antikroppar som motverkar den allergiska reaktionen, ökade som tecken på att tolerans mot allergenen utvecklats.

I den andra studien genomfördes samtidiga intralymfatiska injektioner av björk- och gräsallergen. Behandlingen gav endast milda biverkningar. Effekten mättes med näsprovokation, där gräspollen sprayades in i näsan. De patienter som hade fått aktiv ILIT reagerade med mindre symptom under testet än de patienter som behandlats med placebo. Vidare minskade medicinförbrukningen under pollensäsongen, IgG4 ökade och en typ av vita blodkroppar som verkar inflammationsdämpande, så kallade regulatoriska T-celler, ökade.

Delarbete tre bestod av två studier där vi undersökte om högre doser av ILIT kunde ge bättre effekt. I den första studien gavs ILIT i ett stegrande schema till patienter som nyligen fått konventionell AIT med injektioner under huden, och som därmed redan från början hade ett visst skydd mot allergiska biverkningar. Dosökningen kunde genomföras utan några allvarliga reaktioner och under pollensäsongen därpå hade patienterna mindre symptom och rapporterade en lägre medicinförbrukning. I den andra delstudien testades samma dosökning hos patienter med gräsallergi som inte tidigare behandlats med AIT. Doshöjning upp till 5000 SQ-U (standardiserade enheter) gav akuta, mycket kraftiga allergiska reaktioner som kunde behandlas snabbt men dosupptrappning i resterande del av studien avbröts. Hos de patienter som erhållit en måttlig dosökning upp till 3000 SQ-U sågs överraskande nog ingen förbättring av symptomen.

I det sista delarbetet följde vi upp de patienter som i delarbete II erhållit ILIT mot björk- och gräspollenallergi, 5–6 år efter behandlingen. Näsprovokationen, som året efter vaccinationen visat en reducerad symptomprofil, gav nu åter lika mycket symptom som före behandlingen. Dock var antalet rapporterade symptom och mängden använd allergimedicin under pollensäsongen lägre än hos en motsvarande kontrollgrupp av allergiska patienter som inte tidigare erhållit AIT. Vidare var halten av IgE-antikroppar mot gräs lägre och en typ av vita blodkroppar, så kallade T-minnesceller, i lymfkörtlarna högre.

Sammanfattningsvis belyser den här avhandlingen flera nya aspekter av ILIT. Behandlingen kan ges vid välbehandlad allergisk astma och med två allergen samtidigt. Hos patienter som nyligen erhållit konventionell AIT kan ILIT ge ytterligare symtomförbättring. Dosökning av ILIT utan föregående konventionell AIT är riskabel och verkar inte ge mer symptomlindring än lägre doser. Ökade doser bör därför troligen undvikas. Prover från blod och lymfkörtlar visade att ILIT motverkar några av de mekanismer i immunförsvaret som orsakar allergiska symptom och att det är möjligt att effekten av genomförd terapi varar i upp till 5 år.

Om ILIT kan utvecklas vidare, med mer kunskap om optimala doser och antal injektioner, kan den komma att utgöra ett attraktivt behandlingsalternativ vid AR. Med endast tre sjukhusbesök under en jämförelsevis kort period, skulle ILIT kunna erbjudas betydligt fler patienter än de som idag kan erhålla konventionell AIT, dessutom till en lägre kostnad för sjukvården.

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